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ASTROCYTES: A RESERVOIR FOR NEW NEURONS

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On the cover: In the work presented in this thesis, astrocytes take center stage as the stars of the brain. Indeed, their name even means *star cell*, something I wanted to highlight when making this illustration.

Astrocytes: A Reservoir for New Neurons

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Jens Magnusson

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"How lucky we are to live in this time, the first moment in human history when we are, in fact, visiting other worlds."

- Carl Sagan

ABSTRACT

The goal of regenerative medicine is to be able to help the body to replace worn-out cells and tissues. To achieve this goal, one approach being pursued today is to recruit and boost the body's own mechanisms for cell replacement. In the brain, this approach is perhaps more challenging than in many other organs because the brain's own ability to replace neurons is almost non-existent. Therefore, an alternative strategy is to genetically reprogram support cells in the living brain directly into neurons, or into progenitor cells which in turn would produce neurons. For such a strategy to work, a first step is to identify a cell type in the brain that has the capacity to act as a source for neurons.

This thesis describes work that shows for the first time that astrocytes, one of the most abundant cell types in the brain, can generate neurons *in vivo* under certain circumstances and in certain brain regions. Thus, astrocytes represent a potential reservoir for new neurons, whose neurogenic capacity might be recruited to improve brain repair.

In **Paper I**, we describe the finding that some astrocytes generate neurons in response to stroke in the striatum of mice. We found that the neurogenic capacity of these astrocytes was under control of the Notch signaling pathway. By manipulating this pathway experimentally, we could activate the neurogenic program of these astrocytes even in the absence of stroke. Yet, not all astrocytes generated neurons. In fact, outside the striatum, most of them did not do so, neither after stroke nor after Notch manipulation.

In **Paper II**, we therefore used single-cell RNA sequencing to better understand how astrocytes respond to Notch manipulation and why this does not induce all astrocytes to undergo neurogenesis. We found that even astrocytes that do not generate neurons in fact initiate early steps of neurogenesis. However, they halt their lineage progression immediately before undergoing transit-amplifying divisions. In the striatum, exposure to a mitogen pushed such halted astrocytes into transit-amplifying divisions, and our results suggest that similar strategies could work also outside the striatum, given the right stimulus.

In **Paper III**, we asked whether stroke-induced striatal neurogenesis occurs also in humans. For this, we used radiocarbon dating to assess the age of striatal neurons isolated from post-mortem samples of stroke patients. We found that the stroke-injured striatum contains a higher proportion of young neurons than the non-injured striatum of the same subjects. This could be explained either by neurogenesis or selective death of old neurons. Each of these possibilities would represent an interesting and previously undescribed biological scenario.

The work in these three papers suggests that endogenous brain cells exist whose neurogenic properties could be recruited to improve brain repair. However, one additional challenge with neuronal replacement strategies is that the great diversity of neurons in the brain is still incompletely characterized. Before any cell replacement interventions can be undertaken, the cell composition in the healthy brain must be known. In **Paper IV**, we describe a new method for performing RNA sequencing on intact tissue sections with retained spatial information. This technique, dubbed spatial transcriptomics, is useful for answering fundamental biological questions about cell distribution and gene expression. In addition, it could provide valuable information for disease diagnostics. In summary, the work presented in this thesis provides information that may prove crucial for bringing neuronal replacement strategies closer to the clinic.

LIST OF SCIENTIFIC PAPERS

I. **Jens P. Magnusson***, Christian Göritz*, Jemal Tatarishvili, David O. Dias, Emma M. K. Smith, Olle Lindvall, Zaal Kokaia, Jonas Frisén (2014) A latent neurogenic program in astrocytes regulated by Notch signaling in the mouse. *Science*, 346: 237-241

II. **Jens P. Magnusson**, Jeff E. Mold, Giuseppe Santopolo, Margherita Zamboni, Carlos Talavera-López, Björn Andersson, Ruimin Ge, Zaal Kokaia, Olle Lindvall, Jonas Frisén. Characterization and enhancement of astrocyte neurogenesis based on single-cell RNA sequencing in mice (*Manuscript*)

III. Hagen B. Huttner, Olaf Bergmann, Embla Steiner, **Jens P. Magnusson**, Mehran Salehpour, Elisabet Englund, Carolin Mondorf , Petra Burkardt, Stefan Schwab, Samuel Bernard, Göran Possnert, Jonas Frisén. Dynamics of striatal neurogenesis in response to ischemic stroke in humans (*Manuscript*)

IV. Patrik L. Ståhl*, Fredrik Salmén*, Sanja Vickovic**, Anna Lundmark**, José Fernández Navarro, **Jens Magnusson**, Stefania Giacomello, Michaela Asp, Jakub O. Westholm, Mikael Huss, Annelie Mollbrink, Sten Linnarsson, Simone Codeluppi, Åke Borg, Fredrik Pontén, Paul Igor Costea, Pelin Sahlén, Jan Mulder, Olaf Bergmann, Joakim Lundeberg, Jonas Frisén (2016) Visualization and analysis of gene expression in tissue sections by spatial transcriptomics. *Science,* 353: 78-82

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CONTENTS

1 INTRODUCTION

The brain is not good at repairing itself. This is seen clearly after a stroke. Only minutes after a blood clot or bleeding has obstructed blood flow to the brain, neurons start dying from a lack of oxygen (*1*). These neurons will never be replaced. Instead, the healing brain seals the lesion with a scar, a scar that does not function at all like normal brain tissue.

Stroke is the second most common cause of death in the world, after ischemic heart disease (*224*). But even among survivors, stroke often leads to severe disability and reduced quality of life. As if that was not enough, stroke and other brain disorders caused by neuronal loss place a huge economic burden on society. Such disorders, like Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis (ALS) and traumatic brain injury, have direct health care costs amounting to around €77 billion per year in Europe, which corresponds to 6% of all direct health care costs (*2*). This large economic burden comes partly from the disability and loss of productiveness caused by these disorders. Partly, too, it derives from the fact that brain ailments often cannot be cured but require lifelong treatment. For these reasons, any new therapy capable of replacing lost neurons and restoring brain function, even if only partially, would have a tremendous positive impact, both on individuals and on society.

About two decades ago, it became clear that a few regions of the adult brain contain neural stem cells that continuously produce neurons throughout life. Initially, there was hope that this biological phenomenon represented a hitherto undiscovered mechanism for brain repair. Perhaps these stem cells were capable of supplying the whole brain with replacement neurons, if only their output could be boosted. However, the more adult neurogenesis has been studied, the more it has become clear that newborn neurons are not primarily made for brain repair; instead, they play an important role for some very specialized functions in the healthy brain. The brain will likely not turn into a regenerative organ simply by mobilizing these neural stem cells. This means that other ways must be devised with which to produce new cells for the injured brain. Today, one approach in particular is being vigorously pursued experimentally, namely to genetically engineer support cells inside the living brain, either turning them directly into neurons or first into progenitor cells that in turn generate neurons.

This thesis describes work that represents a small step in this direction – the discovery that astrocytes, an abundant type of support cell in the brain, can generate neurons under certain circumstances in mice. We found that this neurogenic capacity of astrocytes could in some brain regions even be activated in healthy mice through genetic manipulation. This finding introduces the possibility that astrocytes may in the future be used as a source for new neurons.

This work should be understood within the broader context of regenerative medicine. In this rapidly advancing research field, the long-term goal is to be able to replace worn-out cells, tissues and organs throughout the body through biological engineering. However, for this to

become reality, it is necessary to first develop a deep understanding of the mechanisms that govern how the cells in our body normally acquire and maintain their identity, and how our organs are kept functional throughout life in both health and disease.

2 CELL REPLACEMENT AND TISSUE REPAIR

2.1 THE BODY PLAN

Consider the process by which a single cell, the fertilized egg, gives rise to a fully formed body. Through this process – embryo development – hundreds, then millions and finally trillions of dividing cells interact to form highly structured tissues and organs. This happens through an extraordinarily complex process of self-organization: Genetic programs, written in DNA, unfold in every cell at the same time; sequences of genetic subroutines are executed to establish cell identity step by step. All cells may be identical on the genome level, but in the early embryo, minuscule fluctuations may be enough to set even neighboring cells on different paths. Through switch-like genetic circuits, small differences between cells are amplified. Molecules secreted by the jostling and proliferating cells establish the patterning signals that orchestrate the realization of the body plan.

The cells that make up the early embryo have the potential to generate any cell type in the body. These are embryonic stem cells. However, as development proceeds, the potential of these cells gradually diminishes as they divide and differentiate. As the body takes shape, many organs are seeded with what will later become adult stem cells, whose potential is restricted to generating only the specialized cell types of their own organs.

The term stem cell is loosely defined but is generally used to describe cells that display two cardinal properties: the potential to generate multiple cell types, and the capacity to self-renew for an indefinite number of cell divisions. Much of the regenerative potential in adult organs lies in these stem cells. Therefore, strategies to artificially enhance the regenerative potential of the human body might work by recruiting and boosting the output of such cells. For this reason, characterizing the processes by which the body normally maintains proper cell numbers is of utmost importance.

2.2 CELL REPLACEMENT IN THE HEALTHY ADULT BODY

All tissues in the adult body are exposed to some level of wear and tear, and the body therefore needs mechanisms to replace cells that are continuously being lost (Figure 1). In some tissues, the level of cell loss is low or moderate. In these tissues, differentiated cells can often maintain their own numbers through slow but steady cell replication. Examples of cell types that use this mode of renewal include epithelial cells in the lungs and kidneys (*3*, *4*), islet cells in the pancreas (*5*), microglia in the brain (*6*), hepatocytes in the liver (*7*), and likely cardiomyocytes in the heart (*8*). In other tissues, however, the extent of cell loss is so great that special mechanisms have needed to evolve just to be able to maintain the number of cells required for normal function. For example, the bone marrow generates new blood cells at the staggering rate of 200 billion cells per day, which is equivalent to the number of cells in the

Brain
Homeostasis: Neurogenesis only in few neurogenic regions (<0.1% of all neurons replaced over lifetime)
Injury: Very poor to no neuronal replacement. Other cell types replaced more effectively
Origin: Neural stem cells (homeostasis) and, after severe injury in the striatum, facultative stem cells (astrocytes)

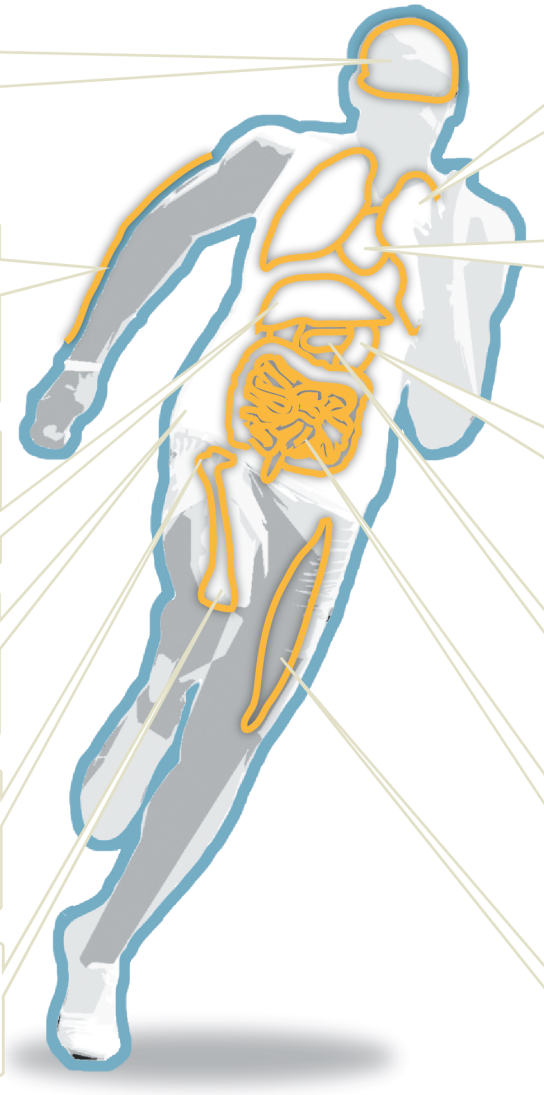
Skin
Homeostasis: Exceptionally rapid turnover of epidermal keratinocytes (5-7%/day)[235 (mice)], sebaceous glands and hair follicles; little to no turnover in sweat glands[24]
Injury: Imperfect wound healing (no new hair follicles, sweat or sebaceous glands)
Origin: Stem cells, some of which expand their potential after injury

Liver
Homeostasis/injury: Great potential for cell proliferation in response to physiological need[7]
Origin: Replication of differentiated cells (mainly hepatocytes)[7]; possibly also facultative stem cells ("oval cells"; debated)[236]

Fat
Homeostasis: 10% of adipocytes replaced per year[242 (humans)]
Injury: Unknown
Origin: Relatively uncharacterized perivascular adipose stem cells[237]; some contribution from bone marrow cells[238].

Other connective tissues (e.g. bone, cartilage, extracellular matrix)
Homeostasis/injury: Tissue deposition and cell replacement in response to need[239]
Origin: Various cells, such as heterogeneous populations of mesenchymal stromal cells and their progeny (e.g. fibroblasts)[239]

Blood
Homeostasis: Exceptionally rapid turnover of most blood cells (up to ~20%/-day for some cell types)[235 (humans)]
Injury (blood loss): Very high regenerative capacity
Origin: Hematopoietic stem cells in bone marrow



Lungs
Homeostasis: Low turnover of lung epithelial cells[3 (mice)]
Injury: High reparative capacity[3 (mice)]
Origin: Proliferation of differentiated cells and, after severe injury, facultative stem cells (e.g. club cells)[3 (mice)]

Heart
Homeostasis: Very low cardiomyocyte turnover (<1%/year)[202 (humans)]; higher turnover of other cell types
Injury: Very poor to no replacement of cardiomyocytes
Origin: Probably proliferation of differentiated cardiomyocytes[8]

Kidneys
Homeostasis: Some cell turnover (e.g. tubular epithelial cells: ~30-50%/year)[240, 241 (mice)]
Injury: High capacity for epithelial cell replacement[4 (mice)]
Origin: Replication of differentiated cells (homeostasis) or facultative stem cells after injury (tubular [and parietal?] epithelial cells)[4, 23 (mice)]

Pancreas
Homeostasis/injury: Turnover of islet cells (0.5-2.0%/day)[235 (rodents)]
Origin: Replication of differentiated cells (homeostasis) or facultative stem cells after injury (ductal cells)[5]

Intestine
Homeostasis: Exceptionally rapid turnover of intestinal epithelial cells (100%/3-5 days)[22 (mice)]
Injury: High capacity for repair of epithelium
Origin: Dedicated stem cells ($Lgr5^+$ cells) and/or facultative stem cells ("+4 cells")[22]

Skeletal muscle
Homeostasis: Unknown
Injury: High capacity for myocyte replacement and repair
Origin: Stem cells (satellite cells)[21]

←**Figure 1. Cell turnover in the adult mammalian body.** Examples of cell turnover in adult organs and tissues are shown. For some cell types, the organisms from which evidence derives are specified in parentheses.

entire human brain (though red blood cells are smaller) and corresponds to about 1% of all blood cells. The situation is similar in the skin and intestinal epithelium, tissues that experience a tremendous amount of abrasion as a result of normal life. In tissues like these, the extreme cell production rates are powered not by the duplication of differentiated cells, but by adult stem cells. When an adult stem cell divides, it can generate one copy of itself as well as one daughter cell termed a short-term stem cell or transit-amplifying cell. This latter cell, in turn, initiates a rapid series of divisions that results in tens of differentiated cells being produced at once, consuming the transit-amplifying cells in the process (Figure 2). Through this process of asymmetric cell division, each stem cell can generate many new cells in one go and avoid depletion of the stem cell pool.

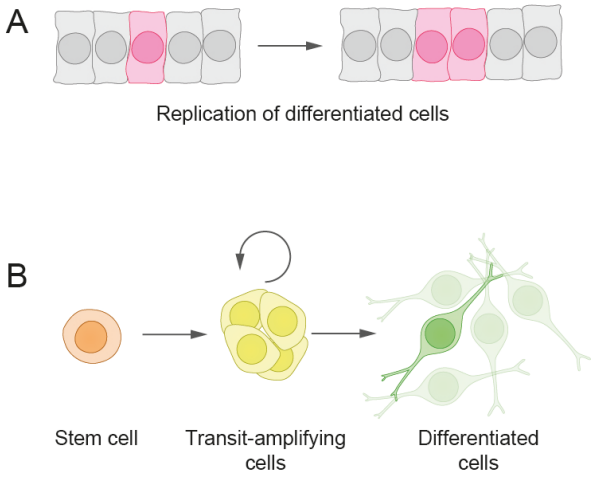


Figure 2. Modes of cell generation in the adult body. Tissues with low to medium levels of cell replacement often rely on the simple duplication of pre-existing cells (A). Tissues with very rapid cell turnover rely on dedicated stem cells, which generate differentiated cells via highly proliferative transit-amplifying cells (B). Figure adapted from (*9*) with permission.

Why have tissues with the highest level of cell turnover evolved this special setup with stem cells? The answer is that it minimizes DNA damage. Every time a cell divides and copies its genome, a few copying errors occur randomly in its DNA. Over the course of many divisions, such mutations accumulate and increase the risk that a cell will transform into a cancer cell that starts dividing uncontrollably. By creating a hierarchy of proliferating transit-amplifying cells, stem cells can generate many differentiated cells at once while at the same time minimizing the proliferative burden they place on themselves. In this way, tissues like the bone marrow, skin and intestinal epithelium can continue to produce new cells and maintain tissue homeostasis throughout life.

Nonetheless, despite this protective mechanism our tissues and organs become less capable of

producing new cells as we age. This decline generally starts already around the age of 30 for some tissues like skeletal muscle, hair, skin, and bone (*10*). That the decline starts so early likely reflects the fact that we humans rarely used to live long beyond our 40s or 50s when our species first evolved; strong evolutionary pressure may not have existed for keeping tissues healthy for many decades more than that. Age-related decline is probably caused partly by the slow but inevitable accumulation of mutations in stem cells. For example, hematopoietic stem cells acquire on the order of 10 mutations per year (*11*). During normal aging, each such stem cell will therefore accumulate hundreds of mutations, scattered more or less randomly throughout the genome. Most of these mutations will have no detectable effect on a cell, and of those that do, most will probably lead to reduced proliferative capacity of the cell. This mutational burden could underlie the decreased capacity to maintain normal cell replacement with age.

Though most mutations are detrimental to a cell's proliferative capacity, some mutations will, purely by chance, endow cells with an increased tendency to divide and survive. In the natural selection-like environment among neighboring stem cells, such mutations give cells a selective advantage. With time, cell clones with the highest proliferative advantage will tend to take over and dominate the stem cell pool. This process, whereby most stem cells decrease their competitiveness but a few increase theirs, is believed to be the cause for an observed decline in stem cell diversity with age (*12*). But what is worse, it may with time cause cells to develop another age-related phenomenon: cancer. This has been demonstrated in the skin (*13*). With age, normal sun-exposed skin develops into a patchwork of dominating cell clones. Strikingly, people in their 50s-70s carry mutations in *TP53*, the most commonly mutated gene among all cancers, in 3-5% of all their skin cells. This corresponds to about 10 cell clones with mutated *TP53* per cm^2 of normal skin. All in all, aged skin contains hundreds of evolving clones per cm^2, each clone consisting of cells carrying thousands of mutations (*13*). These findings are sobering and suggest that over the course of evolution, tissues with very high cell turnover must have been under high selective pressure to evolve mechanisms that minimize the DNA damage associated with cell division. The hierarchies of stem cells and transit-amplifying cells found in tissues like skin, blood and intestine likely represent such a protective mechanism.

Taken together, the mechanisms that replace cells and maintain tissue homeostasis work best for only a few decades in humans, and after that appear more and more unregulated and subject to adverse events. Interestingly, however, one aspect of healthy stem cell maintenance might in the foreseeable future become a target of pharmaceutical manipulation. Stem cells are not only regulated by cell-intrinsic properties but, to an equal extent, also by their extracellular environment. Recent research is showing that the age-related decline of stem cell function derives partly from changes to the extracellular environment. Intriguingly, at least some of these changes may be reversible. In an experiment termed heterochronic parabiosis, researchers have surgically joined the blood vessels of a young and an old mouse, such that the two mice are exposed to each other's blood. These experiments have shown that stem cells in young mice show functional decline when exposed to old blood; conversely,

when the researchers studied stem cells in brain and muscle of old mice, they found that these cells increased their proliferation or regenerative capacity when exposed to young blood (*12*). Now, researchers are trying to identify the factors responsible for this remarkable effect. One emerging conclusion is that molecules associated with inflammation may be culprits. Many such molecules are more highly concentrated in aged blood, reflecting the fact that diffuse, low-grade inflammation is often present in aged individuals (*14*). Such inflammatory factors may impair normal stem cell function. For example, CCL11, a cytokine, decreases hippocampal neurogenesis if infused into the blood of young mice (*15*). Conversely, oxytocin, TIMP2 and, possibly, GDF11 are enriched in young blood and can increase the function of certain aged tissues in mice (*16–21*). The long-term goal of this research is to identify mechanisms and molecules that could be manipulated to reverse the age-related decline in homeostasis seen in most organs.

The mechanisms described here are responsible for the cell replacement that takes place in the healthy adult. However, after injury and in disease, increased cell loss and disruption of normal tissue architecture can often place much higher demands on our body's reparative capacity.

2.3 CELL REPLACEMENT AND TISSUE REPAIR AFTER INJURY

The same mechanisms that serve to replenish cell numbers in healthy tissues are also important for replacing cells lost to injury or disease (see Figure 1). After liver injury, for example, proper cell numbers are restored through the replication of surviving, mature hepatocytes (*7*). In epithelia, such as those in the lungs or kidneys, mature cells successfully repopulate the damaged epithelium through simple cell duplication, at least if the injury is not too severe (*3*, *4*). In a corresponding fashion, tissues where cell replacement normally depends on adult stem cells, the high proliferative capacity of these stem cells soon heal minor abrasions or limited cell loss. For example, even after heavy bleeding, hematopoietic stem cells soon restore normal blood cell numbers.

In addition to these mechanisms, however, injury can trigger modes of cell replacement that are not seen in healthy tissue. If the need for cells is greater than what normal mechanisms of replacement can handle, many tissues recruit dormant stem cells, cells that normally do not display any proclivity for cell replacement. Such cells are sometimes called *facultative* stem cells, the word facultative being defined in biology as "capable of but not restricted to a particular function or mode of life" (*225*). Thus, a facultative stem cell only displays its regenerative potential in the context of injury. In skeletal muscle, satellite cells have this role, reacting to muscle injury by entering the cell cycle and replacing dead myocytes (*22*). In intact muscle, however, they appear to do nothing else than to lie in wait, having no obvious role in normal muscle function. Similarly, the intestinal epithelium harbors "+4" cells, so named for their position as the fourth cells from the bottom of intestinal crypts. These, too,

remain dormant most of the time, but are activated by severe lesions to replace the $Lgr5^+$ stem cells that normally replenish the intestinal epithelium (*23*).

Facultative stem cells are, however, not always silent bystanders in the absence of injury. Often, they lead double lives, having other, unrelated functions in the healthy organ. For example, club cells in lung epithelium, or tubular epithelial cells in kidney, normally have important roles for tissue function, but after severe injuries are able to dedifferentiate and generate also some other cell types (*3*, *24*). In skin, stem cells whose normal lifestyle is restricted to maintaining either hair follicles, sweat glands or sebaceous glands, react to injury by expanding their potential, becoming able to generate also intrafollicular epidermis (*25*). Thus, the cells involved in injury repair have adopted a wide variety of lifestyles. In this thesis, one main finding is the identification of astrocytes as facultative stem cells in the mouse brain, being activated by stroke to generate new neurons.

Ideally, a tissue's response to injury should be to regenerate itself so effectively that it becomes both morphologically and functionally equivalent to what it was before the injury. However, human tissues do not do this. In fact, injury repair in all mammals suffers from three major limitations. The first limitation is that many tissues lack the capacity to replace dead cells effectively. The heart and the brain are particularly bad at this. Most cardiomyocytes that perish during a heart attack are not replaced, and most neurons lost to stroke are never seen again. It is an interesting coincidence that the two organs with the worst cell replacement capacity in the body are also those whose failure kills the most people. As mentioned, the two leading causes of death in the world are ischemic heart disease and stroke. Perhaps because injuries here often lead to instant death, there has not been strong evolutionary pressure for survivors to develop effective repair mechanisms in these organs.

The second limitation to mammalian injury repair is that lesions to almost all tissues result in the formation of a scar. A scar is a mesh of tough extracellular matrix and newly formed cells (*26*). On the one hand, scar formation serves to quickly seal a wound, re-establish tissue integrity, and contain damaging inflammation. This minimizes risk for secondary damage, which the sensitive region surrounding a lesion is susceptible to. On the other hand, scar tissue is not functionally equivalent to the tissue that was lost. Therefore, the function of a healed wound is often reduced compared to what it was before the injury. Scar tissue does not, for example, contribute to the beating of an injured heart. In the skin, scar tissue contains neither sweat glands, hair follicles nor sebaceous glands. And in the intestine, despite the high capacity for cell replacement in its epithelium, any wound that penetrates deeper than that will result in scar formation. This is seen, for example, in chronic inflammatory bowel disorders or after surgical intestinal ligation.

The third limitation to injury repair in mammals is that only relatively small wounds can be repaired. If an entire limb is lost, say, or an eye or a kidney, these cannot be regrown. Once embryo development is complete, the body does not have the capacity to re-initiate the developmental programs responsible for organ patterning. The liver is an illustrative example. This organ has a remarkable capacity to replace lost cells. Even if as much as 75% of the liver

is lost, cells in the remaining part will proliferate until the liver has regained its original size (*27*). However, the shape of the liver will not be the same as before. If one of the liver's two lobes is surgically removed, the regrown liver will contain only one very large lobe. Having now a new, altered shape, it will not be properly held in place by surrounding ligaments (*7*). Thus, the liver is capable of functional but not morphological regeneration.

Therefore, injury repair in mammals is not true regeneration. Instead, the word regeneration could perhaps only properly be used to describe a tissue response that restores both form and function. There are animals where this happens. Among the vertebrates, none display this more spectacularly than the salamanders. This diverse and remarkable group of animals has been studied for their amazing regenerative properties since at least the 1700s, though only in the age of molecular biology have the underlying molecular mechanisms begun to be understood. At the end of the 1800s and beginning of the 1900s, the regenerative capacity of salamanders was extensively mapped through meticulous experiments, many of which would not be considered ethical today (*226*). For example, there are salamander species that can regrow their limbs and tail and regenerate organs such as the spinal cord, jaws, gills, liver, lungs, intestine, eye lens, heart, and brain. Not only are these organs regenerated in function, like the human liver, but also in form, and without scars. Limits exist, however, to their regenerative ability. There must be something left of an organ for it to regenerate. If an entire organ is removed, or if a limb is amputated at shoulder level, they will not grow back. Regeneration requires patterning signals that guide organ growth and cell proliferation, signals that are not properly established if too much of an organ or limb is missing.

The study of salamander regeneration is fascinating, not only as a curiosity, but as an inspiration for what might one day be possible to achieve in humans, given sufficiently advanced tissue engineering technology. The cellular mechanisms that underlie regeneration in these species could in principle be artificially transferred to wound repair in humans. Part of the secret behind the regenerative capacity of these animals lies in their scarless wound healing. In salamanders, cells in a healing wound deposit much less extracellular matrix than in mammals; particularly noteworthy is a different composition of proteins like collagen. In addition, cells in a salamander wound site express matrix remodeling proteins that constantly re-sculpt and remove extracellular matrix, something that does not happen in mammals (*26*).

But perhaps the most striking aspect of salamander regeneration is that lesioned forms a *blastema*, a mass of undifferentiated cells in which molecules secreted by spared nerves and skin establish a signaling center from which patterning signals emerge (*26*)[*]. These patterning signals guide proliferating cells to organize into the proper shape of that limb or organ. A blastema does not form around a wound site in adult mammals.

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[*] Incidentally, a blastema is analogous to the regenerative tissue in plants called *callus*, namely an unorganized mass of undifferentiated cells that forms over a plant wound and serves as the basis for regeneration.

Clearly, then, we mammals lack the regenerative capacity of salamanders. Many of our tissues do harbor a potential to replace lost cells, but after injuries where proper regeneration requires even the slightest bit of patterning, many tissues instead resort to scar formation. This observation prompts the question of whether mammalian cells are fundamentally incapable of mounting a regenerative response similar to that of salamanders, or whether our cells could in principle do it given the right stimuli.

As a matter of fact, examples exist that give cause for optimism. The African spiny mouse (genus Acomys) has evolved a defense mechanism by which the entire skin on its back tears off completely if grabbed. It then quickly mounts a regenerative response to regrow all layers of the skin, including hair follicles and glands (*28*). In the same species, experimental wounds to the ear heal perfectly and without scar formation, through a process that involves the formation of a regenerative blastema.

Even in humans, injuries can heal scarlessly in the fetus (*29*), demonstrating that this is possible in principle. Most spectacularly, though, is the fact that completely severed fingertips can grow back in children if the cut has been distal enough to spare the nail bed (*30*). This fascinating instance of true mammalian regeneration has been studied more closely in mice, which share this capacity (*31*). It turns out that fingertip regeneration in mammals happens through mechanisms that are very similar to limb regeneration in salamanders. Stem cells in the nail bed of the severed fingertip organize a signaling center, which serves to attract nerves. These, in turn, promote the outgrowth of a mesenchymal blastema, from which the fingertip regrows.

Taken together, the wound healing capacity of humans is relatively poor, but on the positive side, our cells do not seem fundamentally incapable of mounting a much more effective regenerative response. It may be possible to achieve improved repair artificially, by tweaking the (admittedly very complex) environment of an injury site to mimic that of a perfectly regenerating wound.

The long-term goal in regenerative medicine is to help the body to replace cell types, regrow tissues and even entire organs. It is inspiring and instructive to study the effectiveness by which these processes happen in some other animal species. Imitating these mechanisms in humans will not be an easy task, but may be possible. Nonetheless, there may be no other part of the body where the goal of tissue regeneration appears more daunting than the nervous system. Here, the sheer complexity of the tissue makes the whole idea of artificially enhanced regeneration seem outlandishly difficult to realize.

2.4 REGENERATION IN THE NERVOUS SYSTEM

As we have seen, the outcome of wound healing is determined by a tissue's capacity for cell replacement, tendency for scar formation and, in severe cases, ability to establish patterning signals that guide re-acquisition of proper tissue architecture. All of these aspects are

important in the nervous system, too. However, due to the special architecture of the nervous system, imperfect wound healing has particularly severe consequences here. This is because neuronal function is completely determined by neuronal connections.

The patterns by which neurons wire up to one another are largely established during brain development. The architecture of neuronal circuits determines which computations these circuits can perform – in other words, which behaviors they are capable of producing. Though some neurons project only locally, others extend their axons over very long distances – many centimeters or even a meter in the case of some of the neurons that innervate the legs. This means that the nervous system is not only susceptible to injuries that kill neurons outright, but also to lesions that only sever their projections.

The nervous system can be subdivided into the central (brain and spinal cord) and peripheral nervous systems (nerves and neuronal aggregates outside the brain and spinal cord). These subdivisions have different developmental origins, and as we shall see, the way they respond to injury is dramatically different.

2.4.1 Regeneration in the peripheral nervous system

In all aspects of repair, the peripheral nervous system appears better than the central (Figure 3). Neuronal projections – axons – provide an important example. These project through the body in bundles called nerves, each axon being wrapped in insulating myelin by Schwann cells. A nerve contains many axons. For example, the two sciatic nerves, which innervate the legs, are the body's thickest. In rats, each contains about 27,000 axons (*227*). The human sciatic nerves, with their diameter of nearly 1 cm, have a cross-sectional area roughly 400 times as big as those of the rat and thus contain many more axons.

If a nerve in the peripheral nervous system gets transected, its axons can regrow to reinnervate their targets. As the distal, dead part of a severed axon is cleared by macrophages, the proximal stump begins to grow across the lesion site, aided by a bridge of dedifferentiating Schwann cells, fibroblasts, macrophages and blood vessels. Schwann cells distal to the injury reorganize themselves and form canals, secreting growth factors that guide the regrowing axons toward their targets (*32*), which grow at a rate of about 4 mm per day in rats (*33*).

This process is remarkable; yet, it is not perfect. Not all axons reinnervate successfully. The slow rate at which axons regrow means that if one is severed very far from its target, it may take months to reach it. The further an axon needs to grow, and the more time it takes, the less likely reinnervation is to succeed (*34*). Moreover, a regrowing axon does not necessarily find its way back to exactly the same target it innervated prior to the injury. Among regrowing axons of motor neurons, for example, some axons succeed and others do not. The ones that do so reinnervate three to five times more muscle fibers than normal, masking the

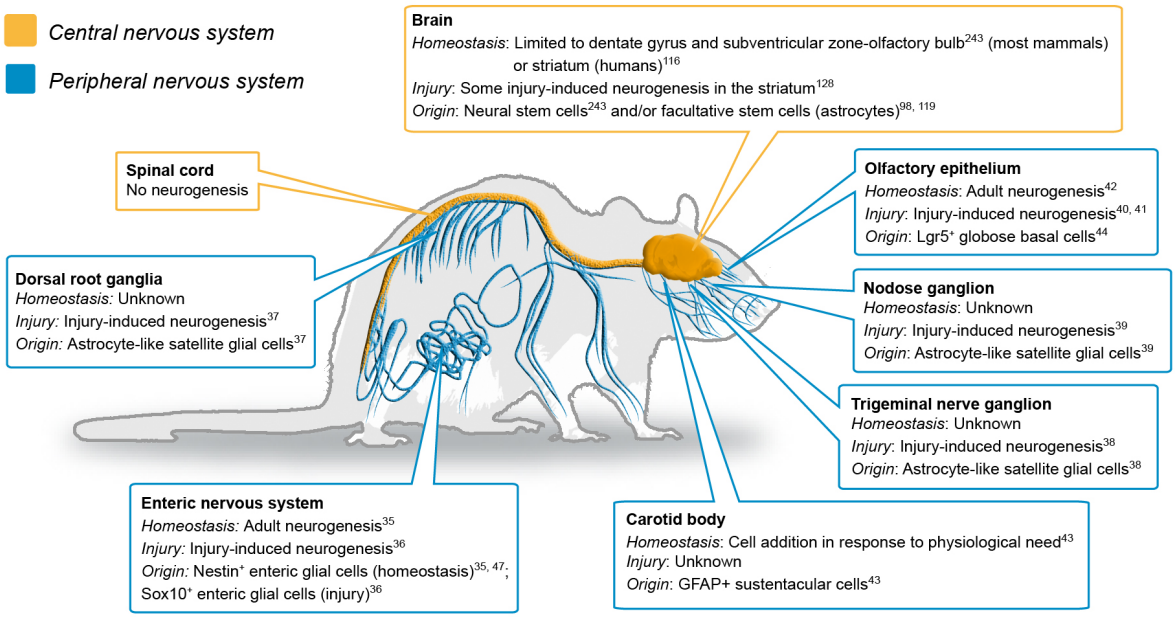

Figure 3. Neurogenesis in the central and peripheral nervous system of mammals. Examples of neuronal cell turnover in the adult mammalian body. Evidence derives from studies in rodents unless otherwise specified.

failure of many other axons (*35*). Notwithstanding these limitations, the fact that axonal regrowth does occur at all in the peripheral nervous system is striking.

Even more noteworthy, perhaps, is that the neurons themselves in the peripheral nervous system can be replaced if they die. An example of this can be seen in the enteric nervous system. This subdivision of the peripheral nervous system runs as a neuronal meshwork along the entire esophagus, stomach and intestine, and to some degree functions independently of input from the brain and spinal cord. Embedded in the muscular layer and submucosa of the gastrointestinal tract, the bodies of neurons are aggregated in clusters called ganglia and plexuses, and consist of both sensory-, motor- and interneurons. Ganglia continuously lose neurons from apoptosis during healthy adult life, but constantly replace them through neurogenesis (*36*). In addition, one study showed that in response to a chemical injury, where a substantial amount of neurons were killed at once, dead neurons could be replaced (*37*). Such injury-induced neurogenesis has been demonstrated also in other subdivisions of the peripheral nervous system, like the dorsal root ganglia (*38*), trigeminal nerve ganglion (*39*), nodose ganglion (*40*) and olfactory epithelium (*41*, *42*).

Neuronal loss is not, however, the only trigger for adult neurogenesis in the peripheral nervous system. The olfactory epithelium displays continuous neurogenesis throughout adulthood (*43*). In addition, neurogenesis can be employed to correct disturbances in some homeostatic functions. The carotid body is a cluster of dopaminergic neuron-like glomus cells in the throat, which detect changes in oxygen and carbon dioxide. In response to a low-oxygen environment, such as that during acclimatization to high altitudes, the carotid body

grows several times its normal size. This expansion is driven by the generation of new glomus cells (*44*). The carotid body is thus similar to the liver in that cell number is precisely tuned to match physiological need.

How are these neurons made? In the peripheral nervous system, neurons are not the only cell types that exist. Myelinating Schwann cells and at least a handful of other cell types similar to the central nervous system's astrocytes are present here. The emerging picture is that such glial cells serve as neuronal precursors. In the carotid body, new cells are generated by the GFAP-expressing sustentacular cells, via Nestin-expressing intermediate progenitor cells (*44*). In the dorsal root ganglia, trigeminal nerve ganglion and nodose ganglia, the astrocyte-like satellite glial cells, expressing GFAP, Nestin and/or Pgp9.5 are the neuronal precursors (*38–40*), and in the olfactory epithelium, $Lgr5^+$ globose basal cells have this role (*45*).

In the enteric nervous system, the neuronal precursors are the enteric glial cells. Like satellite glial cells of peripheral ganglia, these are very similar to astrocytes, both in their marker profile (e.g. GFAP, Glutamine synthetase, Vimentin, S100β) (*46*), as well as morphology and ultrastructure (*228, 229*). They are, however, not identical to astrocytes. One subset expresses Sox10, for example. These $Sox10^+$ cells are responsible for generating neurons in the developing enteric nervous system (*37*), a process to which immigrating Dhh^+ Schwann cell progenitors also contribute in the early postnatal period (*47*). After birth, however, the $Sox10^+$ cells switch from neurogenesis to gliogenesis and do not contribute to adult neurogenesis in the enteric nervous system. Instead, another subset of satellite glial cells, negative for Sox10 but positive for Nestin, steps in and generates neurons during adulthood (*36*, *48*). Still, the $Sox10^+$ cells do not lose their neurogenic potential altogether. They can generate neurons *in vitro* if isolated and cultured (*37*). And after injury they reactivate their neurogenic potential and generate neurons *in vivo* (*37*). Thus, the enteric nervous system appears to have at least two subtypes of enteric glial cells with different roles in adult neurogenesis: The $Nestin^+$ $Sox10^-$ cells, responsible for tissue homeostasis, and the $Sox10^+$ cells, which in the adult only generate neurons after injury and thus represent facultative stem cells. Although the mechanism by which enteric neural stem cells generate neurons is not well characterized, it may be similar to that in the central nervous system and occur through transit-amplifying intermediates: Reports have described cells that express the proneural transcription factor Ascl1 (*37*) or microtubule-associated protein Dcx (*48*), just like developing neurons in the brain. It is not impossible, however, that enteric neurogenesis occurs through a different process whereby one stem cell generates only one neuron at a time. Exactly which types of neurons are born in adulthood, and exactly how they integrate in pre-existing circuits, is not well known in the enteric nervous system. One study showed that the type of neurons generated in response to injury express NPY, nNOS and VIP (*37*), a combination specific to enteric GABAergic interneurons (*49*).

In summary, the reparative capacity of the peripheral nervous system is high; severed axons can regrow and neurons here are replaced both to maintain tissue homeostasis and in response to injury.

2.4.2 Regeneration in the central nervous system

The lifelong capacity of the peripheral nervous system to repair injuries and replace neurons is in stark contrast to the regenerative capacity of the central nervous system, which is dismal. By and large, neurons lost in the brain and spinal cord will never be replaced. Instead, wounds such as those caused by stroke or traumatic brain injury are sealed with a scar, a scar that on the one hand patches up the tissue so that it retains its mechanical strength, but on the other hand does not at all function like normal brain tissue. Because of this, injuries to the central nervous system often lead to lifelong handicaps.

The reason why the brain is so bad at repairing itself could have something to do with the fact that it is so incredibly complex. Its 80-90 billion neurons come in a high variety of subtypes and are heavily interconnected in both local and long-range circuits. Each neuron may have thousands of connections to other neurons. Even if a stroke or another injury damages only a small part, the whole brain may be affected indirectly one way or another. Therefore, repairing even small injuries properly would likely require large parts of the healing brain to re-initiate patterning programs similar to those that guided neural connectivity during brain development. Considering that the brain constantly needs to remain "online", even during a wound healing process, such a thorough repair job may be difficult to pull off. The brain cannot shut parts of itself off for maintenance.

As if this did not make brain repair difficult enough, the repair process must happen quickly. There are two reasons for this. First, the region surrounding a lesion suffers from extensive inflammation. This creates an environment that in itself will kill surviving neurons with time. Secondly, the damaged central nervous system becomes sensitive to mechanical stress because a lesion acts like a rip that disrupts the structural integrity of the tissue. In the spinal cord, an injury that is not patched up quickly tends to tear open from the lesion site, making the wound even bigger (*50*). Therefore, even more neurons risk dying from secondary damage if the healing process takes too long.

Perhaps for these reasons, the brain does not attempt to regenerate lost tissue properly. Instead, it appears to prioritize minimizing the extent of secondary damage. In other words, better to quickly deposit scar tissue that encapsulates inflammation and re-establishes tissue integrity than to risk an extended healing process. A poor regenerative capacity might be the price our brain pays for its complexity.

Neuronal replacement after injury does happen in some species. In salamanders, experimental selective ablation of dopaminergic neurons leads to replacement of these neurons (*51*, *52*). Larger physical lesions, too, can heal successfully in some species. In fish, experimental stab wounds regenerate seemingly perfectly (*53*). And in the salamander, the brain will regenerate after the experimental removal of as much as a third of one hemisphere (*230, 231, 232*), and recreate all neuronal subtypes in their correct proportions (*54*). This repair capacity is astonishing. But even in salamanders, there are limitations to the brain's regenerative capacity

after such very large lesions. Newly generated neurons fail to organize themselves properly. And although they successfully establish short-range neuronal connections, connections that extend a millimeter or more are not re-established (*54*). This imperfect brain regeneration in salamanders is a reminder of the fact that tissue regeneration is not only about recreating lost cell types; it is equally much about re-establishing correct tissue patterning signals that help a healing organ re-acquire its proper shape. Failure to do so will result in a repaired organ that is not identical in form to what it was before an injury. This is true for all organs, but perhaps demonstrated most clearly in the central nervous system. Here, organ function is intimately tied to organ shape because of the importance of proper neural connectivity.

An important aspect underlying limited repair capacity in the central nervous system is that its neurons are very bad at regrowing their axons, in contrast to those in the periphery. This is partly due to an environment non-permissive to axonal regrowth. After injury, a scar forms a chemical barrier of inhibitory molecules (*55*), which effectively block axons from penetrating the injured region. But even in the absence of injury, the central nervous system is refractory to axonal growth. This is demonstrated by experimental observations of axons belonging to peripheral neurons that try to grow into the healthy spinal cord. Immediately upon reaching the boundary between the peripheral and central nervous systems, these axons promptly stop growing (*56*). One reason for poor axonal regrowth in the central nervous system is that axons here are not myelinated by Schwann cells capable of guiding axonal regrowth, but by oligodendrocytes, which do not have this guiding role. This guiding role may be crucial. In fact, neurons of the central nervous system can show some capacity for axonal regrowth: Lower motor neurons, whose cell bodies reside in the spinal cord but whose projections extend into the periphery, can regrow their Schwann cell-ensheathed axons if these are transected in the periphery (*57*). Yet, the ability of severed axons to form a growth cone, a necessary appendage, is invariably worse in in central neurons than in peripheral ones (*58*). Instead of forming a proper growth cone, neuronal subtypes that are incapable of axonal regrowth tend to form a retraction bulb, which contains a disorganized microtubule network. This tangle disables the capacity for further axonal outgrowth (*59*, *60*). It must be mentioned that long-range axonal regrowth has been shown to occur in the central nervous system in some cases. For example, one study found that after a cortical lesion in rats, neurons from the intact contralateral cortex projected long-range axons (millimeters) to the de-innervated striatum in the injured hemisphere (*61*).

Taken together, the central nervous system's capacity for injury repair is deplorable, both when it comes to neuronal cell replacement and axonal regrowth. Notwithstanding these limitations, however, the brain is in fact incredibly plastic in other ways. This is of course seen clearly in the process of normal learning, which mechanistically is a remodeling of dendrites and synapses. This type of plasticity becomes particularly high after brain injury, such as a stroke. Here, neurons in intact, neighboring brain regions can rewire their local connections so that spared regions take over functions of lost regions, at least to some extent (*62*). This type of plasticity is responsible for the limited functional recovery seen after stroke.

Also, although most neurons are never replaced after injury, the brain is not completely impervious to cell replacement. Only half of all cells in the human brain are neurons (*63*). The other 50% are cells of other types: astrocytes, oligodendrocytes, oligodendrocyte progenitor cells, ependymal cells, microglia, pericytes and endothelial cells. Such cells do get replaced, even in the healthy adult brain. For example, the cell types that have been studied in humans – oligodendrocytes and microglia – turn over in the healthy cortex at a rate of 2.5% and 28% per year, respectively (*6*, *64*). Studies in rodents have shown that in response to injury, also astrocytes, oligodendrocyte progenitor cells, ependymal cells, pericytes and endothelial cells can enter the cell cycle and at least to some extent maintain their own numbers (*65–69*). This suggests that poor neuronal replacement is not caused by the brain being generally hostile to cell replacement, only to that of neurons.

2.4.3 Neuronal cell replacement through transplantation

It might be possible to improve brain repair if neurons could be supplied from outside through transplantation. An attractive target for such a therapy is Parkinson's disease, as this disease is characterized by the loss of a specific neuronal subtype (dopaminergic neurons) in a specific, small region of the brain (the substantia nigra). Indeed, studies in rodents have shown that transplanted dopaminergic neurons exert functionally beneficial effects in experimental models of Parkinson's disease (*70*, *71*). Therefore, it may in principle be possible to cure this disease in humans by replacing lost neurons through transplantation. Already in the 1980s and 1990s, clinical trials were made with this approach in patients afflicted with Parkinson's disease (*72*). Here, neuronal precursor cells from the midbrain of aborted fetuses were transplanted to 300-400 patients with the hope that these cells would mature into functional dopaminergic neurons once they reached their target brain region. The results were varied. Some patients improved so much that they could be taken off of their normal medications. Others showed little or no effect from the treatment, and some even developed dyskinesias, involuntary movements, as a response to the treatment. Postmortem analysis of some patients, years after transplantation, showed that transplanted cells could indeed survive and integrate into neuronal circuits. Because of these promising but varied results, new and very similar trials are now being performed, this time using more stringent patient selection and characterization of transplanted cells (*233*). Cell transplantation has also been tried for other brain disorders, such as stroke, in early clinical trials. So far, however, such interventions have failed to show any robust improvement in patients (*73*).

It is likely that the outcome of transplantation interventions will improve in the future. This will partly be due to our increased ability to characterize and produce the specific neuronal subtypes that are needed. Today, advances in RNA sequencing technology are enabling the characterization of all cell types that exist in the brain and are lost to injury. This will guide the derivation of specific cell types needed for successful transplantation. Furthermore, advances in cell reprogramming technology is improving the capacity to generate correct neuronal subtypes in vitro (*74*). For example, very exciting progress is being made on

generating neurons in a culture dish – cells that could in theory be used for transplantation. These cells have so far been generated largely from fibroblasts or astrocytes, either through forced expression of transcription factors (*75–85*), which can be aided by the addition of small molecules (*86–88*), or by only using small molecules and no gene overexpression (*89–93*).

3 NEUROGENESIS IN THE ADULT BRAIN

3.1 ADULT NEUROGENESIS – THE CONTINUED GENERATION OF NEW NEURONS THROUGHOUT ADULTHOOD

Considering the difficulties with cell transplantation, it caused great excitement when it became clear in the 1990s that there are in fact some regions of the brain where new neurons are being generated throughout adulthood. In hindsight, the evidence for this had been slowly accumulating for decades. In the 1960s, Joseph Altman and Gopal Das published their finding that cells in the adult rat dentate gyrus incorporated radioactive thymidine – a sign that these cells were adult-born (*94*, *95*). Maybe these cells were neurons, maybe not; the experimental methods were not good enough to tell. More than a decade later, Michael Kaplan replicated Altman's experiment but now used electron microscopy to show that the adult-born cells were indeed neurons (*96*). Almost another decade later, Fernando Nottebohm demonstrated that adult-born neurons are incorporated into functional neuronal circuits, though this was not shown in mammals but in canary birds, whose seasonal song learning is accompanied by bursts of neurogenesis (*97*). Then, in 1992, a number of research groups showed that it was possible to isolate neural stem cells from the adult rodent brain and expand them *in vitro* using growth factors (*98*, *99*). This was followed by studies confirming that adult neurogenesis does in fact take place in the living mammalian brain. In 1998, it was shown for the first time that this process also occurs in the adult human brain (*100*) (Figure 4).

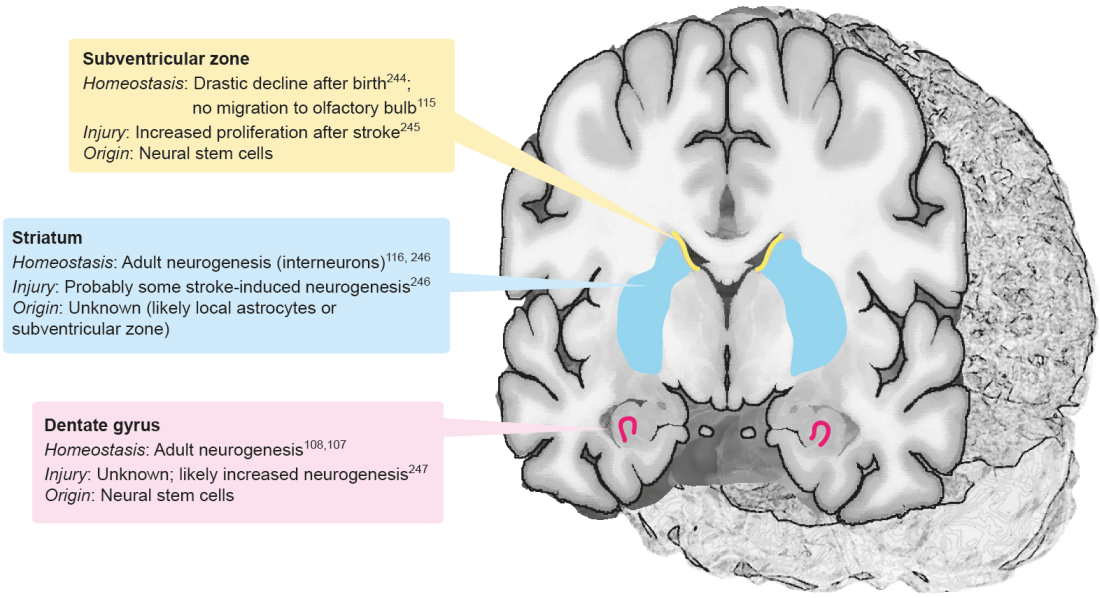

Figure 4. Neurogenesis in the adult human brain.

This story, spanning over 30 years, may seem like excruciatingly slow scientific progress. But it is only with the luxury of hindsight we can see that these pieces of evidence were pointing to something that was real. The understanding up until this point had been that no neurogenesis takes place after development, and there was no clinical evidence whatsoever to suggest otherwise. It is often said that the discovery of adult neurogenesis broke a long-held "dogma" claiming that adult neurogenesis was impossible. But the prevailing view was not dogmatic in the sense of a doctrine laid down by an authority. Rather, the notion of adult neurogenesis must have appeared at the time as an extraordinary claim, which as always, requires extraordinary evidence. Scientists are a critical bunch and the initial findings were most likely met with appropriate skepticism. Unlike other important scientific discoveries – say, the structure of DNA – the first reports of adult neurogenesis did not immediately provide an explanation for some well-recognized mystery. If that had been the case, the scientific community might have been quicker at accepting these results. Instead, the discovery of adult neurogenesis preceded the questions it would provide the answer for (*234*). Therefore, the existence of this phenomenon was not broadly accepted until experimental methods developed to the point where the evidence for it became undeniable. Today, the study of adult neurogenesis has developed into an entire field of its own, with hundreds of articles being published each year (Figure 5).

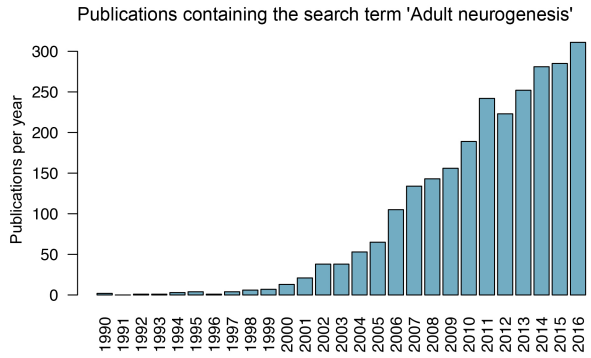

Figure 5. Number of articles about adult neurogenesis published per year. A diagram showing the number of peer-reviewed publications containing the search term "Adult neurogenesis" published per year (source: PubMed). Note that the drastic increase started at the end of the 1990s, when it became clear that adult neurogenesis occurs in the human brain.

Defining which brain regions are 'neurogenic' and which are not may sound like it would be straightforward once people realized that they should look for it. But it is not that trivial to map the neurogenic capacity of the brain. Indeed, more than two decades on, it is still possible to publish a thesis on the subject. The main reason for this is the same as for the study of cell turnover in the rest of the body, namely that an organ may have facultative stem cells that do not always display their cell-producing capacity. To identify adult stem cells, therefore, a cell type of interest must be labeled at one time point and its behavior observed at a later time point. Because this is experimentally demanding, neural stem cells are most often studied in animal models. Laboratory animals may or may not be representative of humans. Yet another difficulty in the field of neurogenesis deals with the definition of 'neurogenic'. Must a neurogenic region display a continuous production of neurons? Is it enough that its

cells have an intrinsic capacity to generate neurons even though this capacity is revealed only in cell culture (e.g. the rodent cortex)? Or is the main feature of a neurogenic region that it has an environment permissive for incorporating new neurons, even though it does not itself have any active neural stem cells (e.g. the rodent olfactory bulb)?

Regardless of how a neurogenic region is defined, however, two parts of the mammalian brain clearly stand out – the dentate gyrus of the hippocampus and the subventricular zone lining the lateral ventricles. In these regions, neural stem cells generate more than a thousand new neurons every day in adulthood (*101*, *102*). When this became firmly established, there was much hype around the possibility that adult neurogenesis might represent a previously undiscovered mechanism for brain repair. Perhaps the purpose of neurogenesis was to act as an intrinsic mechanism for brain repair that could be tweaked to work better? Yet, the more the phenomenon was studied, the more it became clear that newborn neurons instead play important roles for the normal function of the healthy brain. It turns out that young neurons are required for some neuronal circuits to perform certain computations. The most studied neurogenic region is the hippocampus, a brain structure important for learning and memory formation. This region can be described as a "gateway to memory": Information that reaches the hippocampus, such as sensory information, is compressed, catalogued and sent on to the cortex where it is stored as memories. Thus, the hippocampus is not itself the place where memories are stored; rather, it acts like a librarian that makes sure that the right memory is stored in the right place and can be accessed when needed. Hippocampal neurogenesis is confined to a subregion called the dentate gyrus, which is placed like a bottleneck in the circuit through which input to the hippocampus must pass. Through neurogenesis, the dentate gyrus constantly maintains a pool of young neurons. Notably, newborn neurons have distinct electrophysiological properties for some time after they are born, which old neurons do not have: They are hyper-excitable and thus require weaker stimulation in order to get activated (*103*). This property appears to be important for the brain's ability to learn new things. When we memorize new information (such as a text on how memory formation occurs), information travels through the brain in neural circuits that are not so well established. This makes the input to the hippocampus weak. Newborn neurons may help the hippocampus detect these faint signals and thus to process and store the new information as unique memories that are distinct from previously existing memories (*104*).

In the other neurogenic region, the subventricular zone, most mammals generate immature neurons that migrate forward through the brain to the olfactory bulb, where they are integrated as mature neurons. Here, the functional role of newborn neurons is less well understood, but is related to certain aspects of odor discrimination (*105*).

Adult neurogenesis may be described as a mechanism to replace old neurons with new ones, but it is worth noting that neuronal replacement should not be understood as one neuron dying and another coming in to take its exact place. Rather, it should be seen as a balance between the two parameters cell birth rate and cell death rate. Depending on how these parameters are tweaked, the consequences for the tissue are different. In the mouse olfactory

bulb, the neuronal death rate is high and neurogenesis is important for maintaining proper numbers of neurons. If neurogenesis is experimentally ablated, the olfactory bulb gradually deteriorates as neurons die off (*106*). In contrast, the mouse dentate gyrus has a very low neuronal death rate. Through neurogenesis, therefore, the total number of dentate gyrus neurons increases throughout life (*106*). In the dentate gyrus of humans, however, the situation appears to be slightly different. Here, even though neurogenesis happens at the same rate as in middle-aged mice, the neuronal birth rate appears unable to keep up with neuronal death rate. This means that the total number of dentate gyrus neurons declines throughout life (*101*).

Not surprisingly, perhaps, there are differences in the extent of adult neurogenesis between mammalian species. In humans, a larger proportion of dentate gyrus neurons are replaced over a lifetime than in rodents (*101*). Whales and dolphins, on the other hand, barely even have a hippocampus and no neurons are generated there (*107*). Even more strikingly, however, is that humans, unlike other mammals, do not have any adult neurogenesis in the olfactory bulb (*108*). Perhaps this odd absence reflects the fact that the sense of smell is not as important in humans as it is in most other mammals. Another possibility is that the sheer size of the human brain would require neuroblasts to migrate such long distances to reach the olfactory bulb that they cannot do it (as discussed in Section 3.3.1).

A general concept that has dominated the field proposes that, throughout evolution, the capacity for neuronal replacement has decreased with increasing brain complexity. This idea likely originates in comparisons between mammals and animal groups like amphibians, reptiles, and fish, in which mammals indeed have the worst regenerative capacity after injury. This "rule" was thought to apply even between different mammalian species. In effect, the common but questionable assumption that the human brain is more 'complex' than that of rodents has led to the general belief that humans likely have less neurogenesis than mice. For this reason, the discovery that adult humans have ongoing neurogenesis also in the striatum came as a surprise (*109*). Mice, which are the most commonly used laboratory animal, do not have neurogenesis in this region under normal conditions (*110–113*), except during a short period after birth (*114*). However, only because mice are the most commonly studied model organism, we must not therefore think that this species best represents the human brain. Humans are, for example, equally distantly related to rabbits, which do have striatal neurogenesis (*115*). In addition, some reports have claimed to detect striatal neurogenesis even in healthy rats and a non-human primate (*116–118*), though this is controversial (*119*, *120*).

The functional role of new neurons in the striatum is unknown. However, adult striatal neurogenesis appears to be limited to the medial striatum both in rabbits, rats and squirrel monkeys (*115*, *116*, *118*). This brain region receives input from limbic emotional circuitry and the visual and auditory cortex, but not extensively from the motor cortex, which projects primarily to the lateral striatum (*121*). In the healthy striatum, turnover appears to be limited to a population of calretinin-expressing interneurons (*109*, *115*, *122*), though other neuronal

subtypes may be generated after injury, at least in rats (*123*). In humans, 10% of striatal neurons belong to this interneuron subtype, but in mice, only 1% do. It is possible, therefore, that this subtype has a particularly prominent role in the human brain, something that might help explain why they turn over in humans but do not in mice.

In addition to the brain regions mentioned here, there are occasional reports of newborn neurons in other healthy regions. Mostly, though, these studies are based on ambiguous images of single cells and have in general not stood the test of time. Of interest, however, is that for a short time after birth, some level of neurogenesis appears to persist in the hypothalamus (*124*), meninges (*125*), and cerebellum (*126*, *127*), at least in mice.

Though adult neurogenesis does not seem to have evolved as an intrinsic tool for brain repair, many studies describe that there are changes in neurogenesis in response to various injuries. The most well-described of these is that a great surge of neurogenesis develops in the rodent striatum in response to severe injury, particularly stroke. In the weeks and months following a stroke, large amounts of immature neuroblasts appear here. At least some of these neuroblasts mature and integrate as functional striatal neurons (*128*). However, they are few: One study in stroke-injured rats found that after six weeks, only 0.2% of dead striatal neurons had been replaced (*117*). In addition, the type of neurons generated in this way may not be the same as the types that were lost (*122*), though there may be species-specific differences here, even between mice and rats (*123*). In this thesis, Paper III describes work suggesting that stroke-induced striatal neurogenesis occurs also in humans.

Outside the striatum, injury-induced neurogenesis is extremely restricted, to the degree that it would not seem to be of any value for functional recovery. The cortex is a good example. Although some research groups have reported isolated newborn neurons in the rodent cortex injured by stroke or selective neuronal loss (Reviewed in *(234)*), other groups have not found any such neurons (*129–132*). A recent study that used radiocarbon dating to estimate the age of neurons in the human cortex of stroke patients found no evidence whatsoever for neuronal replacement (*133*).

Interestingly, however, a recent study demonstrated that a population of agouti-related protein (AgRP)-positive hypothalamic neurons located outside the blood-brain barrier are replaced after selective ablation of these cells induced by monosodium glutamate injections in mice (*134*). This is fascinating, as this form of injury-induced neurogenesis is reminiscent of that happening in the peripheral nervous system in response to damage.

In summary, adult neurogenesis is highly restricted to a few brain regions, both in health and after injury. Why should this be so? What is so special about these neurogenic regions and the cells located there?

3.2 NEURAL STEM CELLS AND THEIR IDENTITY AS SPECIALIZED ASTROCYTES

The neurons that originate in the dentate gyrus and subventricular zone are made by stem cells (Figure 6). These neural stem cells are similar to those that exist in other organs, like the skin, blood and intestinal epithelium, in that they produce cells through a hierarchy of proliferating intermediate cells. Each time a neural stem cell divides, it generates a copy of itself as well as a transit-amplifying cell, the latter of which undergoes 5-6 additional doublings in rapid succession to generate many neuroblasts. As we have seen, such a hierarchical arrangement is common in organs where high cell turnover is crucial for normal tissue function. Since it evolved also in the neurogenic niches, it suggests that the mechanisms underlying neurogenesis have been under high selective pressure. It is testament to the importance of lifelong neurogenesis for normal brain function.

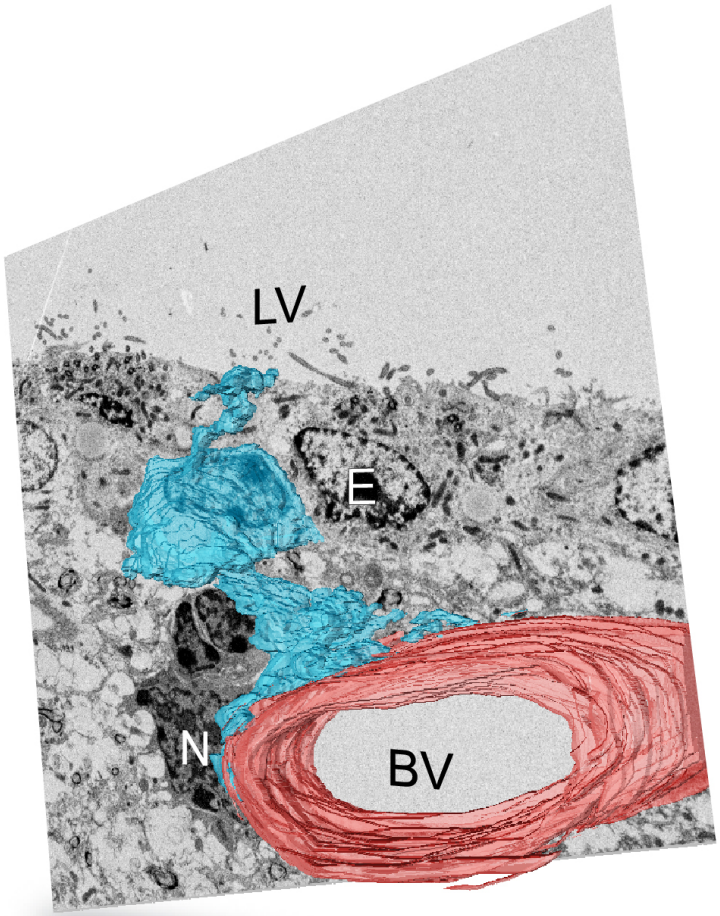

Figure 6. Neural stem cell in the subventricular zone. 3D reconstruction of serial electron micrographs showing a neural stem cell (blue) in the adult mouse subventricular zone. Note the characteristic apical process, which contacts the cerebrospinal fluid in the lateral ventricle (LV), and the basal process, which contacts a blood vessel (BV, red). Also visible in the picture are three ependymal cells (E) and four neuroblasts (N).

One question fundamental to stem cell biology is how a single population of stem cells can generate a diverse population of mature cells. In the subventricular zone, neuroblasts develop into a large variety of olfactory bulb neurons (*135*). Is it the case that each individual stem

cell is flexible enough to generate many different cell types, or are the stem cells in fact as diverse as the cells they give rise to? And can an individual stem cell keep dividing throughout life, or do stem cells take turns to proliferate?

Traditional definitions of stem cells call for two fundamental properties: multipotency, which is the capacity to generate more than one cell type, and self-renewal, which is the ability to maintain multipotency over an indefinite number of cell divisions. If adult neural stem cells are isolated and cultured *in vitro* in the presence of growth factors, they do display these properties (*98*, *136*). However, in their natural environment in the living brain, multipotency and self-renewal might actually only emerge on the population level. Take multipotency, for example. In the subventricular zone, individual stem cells produce either neurons or oligodendrocytes, but not both (*137*). Indeed, each stem cell may even be restricted to producing only a specific subtype of neuron (*138*). The situation might be similar for self-renewal. Stem cells in the subventricular zone may spend very long periods of time in latency. When finally activated, they go through only one or a few quick bursts of proliferation, after which they fall back into an exhausted dormancy that may last for the rest of the animal's life (*139–141*). This means that continuous, lifelong neurogenesis may in this region be the result of many stem cells taking turns to proliferate.

It is possible that both multipotency and self-renewal work differently in the dentate gyrus. Here, some individual stem cells can generate not only neurons but also astrocytes (*142*). Whether each stem cell is also capable of sustained self-renewal is still a matter of debate. One study found that dentate gyrus stem cells can enter and exit an activated state more than once (*142*); another study, in contrast, found that stem cells are exhausted after only a single proliferative burst (*143*). The reason for this disagreement is not well understood.

Regardless of the details, however, it is clear that neural stem cells can display greater potential *in vitro* than what their *in vivo* environment allows them to do. This reflects the by-now well recognized fact that a stem cell's intrinsic potential cannot be discussed without considering the microenvironment that surrounds it – its niche. Environmental signals reach stem cells in the form of growth factors and neurotrophins, neurotransmitters and receptor ligands of various kinds, interactions with the extracellular matrix, neurons, ependymal cells, immune cells, astrocytes, a highly organized vasculature, and the stem cells themselves (*144*, *145*). These signals feed directly into intracellular signaling circuits and prop up the stem cell state.

The realization that the neural stem cell state is a product of both cell-intrinsic and -extrinsic factors is highly interesting. Cells with latent stem cell capacity – facultative stem cells – are common throughout the body but can be difficult to identify as stem cells because they display their stem cell properties only under certain circumstances, usually after severe injury. This introduces the possibility that facultative stem cells may also exist in the adult brain – cells whose stem cell potential is revealed only when the environment is right. What might the identity of such cells be?

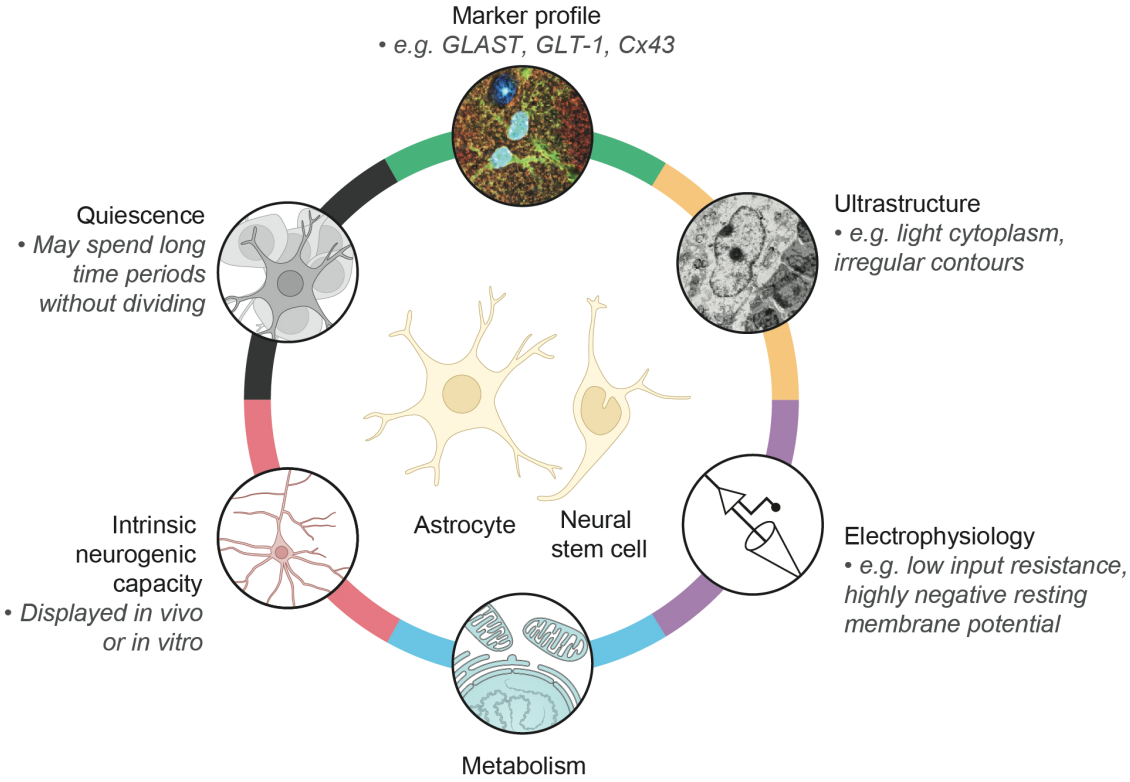

Figure 7. Features shared between astrocytes and neural stem cells. Parenchymal astrocytes and adult neural stem cells have many properties in common. They are similar on the transcriptional level and express many of the same marker genes, they have similar ultrastructural and electrophysiological features, and they are metabolically similar (e.g. low lipid metabolism in their ground state), though the latter is largely inferred from RNA sequencing studies (*146*). They even share an intrinsic capacity to generate neurons, though astrocytes do not normally display this capacity. Instead, astrocytes may spend long periods of time in quiescence, which in fact many neural stem cells do, too. Figure reproduced from (*9*) with permission.

In the dentate gyrus and subventricular zone, the neural stem cells are in fact specialized astrocytes (**Figure 7**). This is demonstrated by the fact that the transcriptomes of astrocytes and neural stem cells are very similar (*146*). For example, neural stem cells express astrocyte-specific genes such as Cx30, GLAST (Slc1a3) and GFAP (*112*, *147*). In addition, astrocytes and neural stem cells share ultrastructural features, as seen using electron microscopy (*148*, *149*), and electrophysiological properties (*150*).

The fact that neural stem cells are specialized astrocytes warrants a closer look at the properties of the astrocytes that occupy the rest of the brain, outside the neurogenic niches. In the human brain, about half of all cells are neurons and the rest is a mix of glial cells and other cell types. Astrocytes are distributed throughout the central nervous system. In the mouse, they make up 10-20% of all brain cells (*151*). They are, however, not evenly distributed throughout the brain. In the mouse cortex, hippocampus and spinal cord, 12-17% of all cells are astrocytes, whereas the corresponding number in the cerebellum and olfactory bulb is only 3-4% (*151*). Astrocytes are crucial for regulating many homeostatic functions in

the brain, including synaptic transmission, ion transport and regulation of pH and the blood-brain barrier. In addition, they mount an important response to injury known as reactive gliosis.

The similarity between astrocytes and neural stem cells is not limited to gene expression and morphological properties. In fact, it also encompasses an intrinsic capacity to generate neurons. If astrocytes are isolated from the injured brain parenchyma and cultured *in vitro* in the presence of growth factors, they activate stem cell-like properties and generate neurons in the culture dish (*152–154*). Now, we and others have found that some astrocytes can even generate neurons *in vivo* after certain injuries (*112*, *113*). These findings are described in this thesis.

3.3 STIMULATING ENDOGENOUS NEUROGENESIS

The realization that some brain regions continuously generate neurons suggested that neurogenesis might be recruited for therapeutic purposes, to replace neurons lost to injury or disease throughout the brain. For this, two general strategies have been and are being pursued, both of which have strengths and weaknesses.

3.3.1 Stimulating neuroblast migration from the subventricular zone

The most straightforward strategy may be to recruit the stem cells in the brain's neurogenic regions, boost their output and direct the migrating neuroblasts to an injured brain region. Normally, the rodent subventricular zone may only be able to generate neurons for the olfactory bulb; however, in response to stroke there is increased proliferation and neurogenesis, followed by neuroblast migration into the injured striatum (*117*, *155*, *156*). This shows that neuroblasts do have the ability to migrate through the brain parenchyma toward a lesion, thus making the strategy feasible in theory.

Neural stem cell proliferation in the subventricular zone can be boosted artificially, even in the absence of severe injury, by injecting growth factors such as EGF, FGF2 or TGF-α directly into the lateral ventricles or the striatum of rodents. However, this on its own does not lead to neuroblast migration into the striatum unless it is performed together with an experimental injury, such as stroke or a 6-OHDA lesion, or in an R6/2 mouse model of Huntington's disease (*110*, *157*, *158*). This is because the critical guidance cue needed to direct neuroblasts into the brain parenchyma appears to be the inflammation caused by injury. Striatal injection of lipopolysaccharide (LPS), a bacterium-derived molecule that causes strong inflammation without causing great injury, is as effective as stroke at promoting migration of neuroblasts into the striatum (*159*). This neuroblast attraction is mediated at least in part by the inflammatory cytokines CXCL12 (SDF1) (*160–162*), CXCL13 (*159*), and CCL2 (MCP1) (*163*), as well as Osteopontin (*164*) and Angiopoietin (*165*), at least in

rodents. It is not known whether it would be possible to stimulate neuroblast migration in humans in the same way.

This approach may sound straightforward, but it has a number of problems. First of all, neural stem cells may be restricted to generating only particular types of neurons. Even after a stroke has affected the striatum, new-made striatal neurons are mostly interneurons, which constitute a minority of all striatal neurons. It is not known whether subventricular zone stem cells have the capacity to generate the full complement of neuronal subtypes that need to be replaced after an injury.

Secondly, from the perspective of a migrating cell, the distances in the brain are very large, and brain tissue is tightly packed with cells and extracellular matrix. In order to squeeze themselves forward through this dense medium, neuroblasts first project filaments that dynamically probe their surroundings. Then, the entire cell drags itself forward within one of these filaments – a bit like how an earthworm crawls (*156*, *166*). To help in this process, they secrete matrix metalloproteinases that break down extracellular matrix in their path (*167*). Neuroblasts prefer to migrate along blood vessels, perhaps because this is faster. Even so, they are relatively slow, migrating through brain tissue at a speed of only about 12 μm per hour (*168*). This means that even if the subventricular zone would in principle be capable of producing neurons for the whole brain, the sheer distances that neuroblasts would need to traverse are huge. For example, a stroke injury located only 5 cm away from the subventricular zone would take 170 days for a neuroblast to reach, even if it migrated in a perfectly straight line, which it most likely would not do. By that time, the stroke lesion would have already reached the chronic phase and it is not certain whether it would still be secreting the factors needed to attract neuroblasts, or indeed whether there would still be any use for new neurons in circuits that had already had half a year to adapt in other ways. For this reason, the subventricular zone could probably only be a useful source for new neurons if injuries are very near. For all other brain regions, any endogenously produced neurons must likely come from more local sources[*].

3.3.2 Reprogramming of glial cells

Another idea for how to generate new neurons within the brain is to recruit local non-neurons as a source for these cells. This idea derives primarily from two separate lines of evidence. The first is that glial cells can be isolated from non-neurogenic regions of the brain, and pushed to generate neurons *in vitro* when stimulated with the right growth factors (*152–154*). This means that the brain is in fact full of cells with an intrinsic potential to generate neurons, though these cells never realize this potential in the living brain. In rodents, these cells are

[*] Similarly, as mentioned in Section 3.1, brain size could also be the reason we humans have lost olfactory bulb neurogenesis, as this would require neuroblasts to migrate very far from the subventricular zone.

astrocytes (*153*) and ependymal cells (*169*) but not oligodendrocyte progenitor cells (NG2 cells) (*170*).

The other line of evidence is the discovery that virtually any differentiated cell in the body can be reprogrammed to a pluripotent stem cell, which in turn can be made to differentiate into any cell type of interest (*171*, *172*). This discovery was awarded with the Nobel Prize in 2012. In principle, this means that every cell in our body is a potential stem cell with the highest developmental potential. In reality, though, cells do not undergo such transformations in the body because their identities are firmly locked into place by epigenetic mechanisms and transcriptional networks (*173*, *174*). Instead, to reprogram cells like this requires the artificial expression of key genes. This is in principle possible in the brain. However, the fate of pluripotent stem cells cannot easily be controlled. Therefore, it would be more desirable to generate neurons through a more direct route. This, too, is possible. In fact, it can be easier to reprogram one differentiated cell type directly into another cell type, especially if the two are closely related. This is because similar cells have similar transcriptomes (*175*), and smaller perturbations may therefore be needed to convert one into the other.

Together, these two lines of evidence show that there are cells in the brain that are very closely related to neural stem cells, and that relatively small genetic manipulations may be sufficient to turn them into neural stem cells, or even directly into neurons. Current research suggests that this is a realistic goal to pursue, and great advances have been made to this end during the past decade. This *in vivo* reprogramming could in principle be achieved in two ways – either through the direct conversion of each starter cell into one neuron, or by first converting each starter cell into a neural stem cell-like cell that in turn generates neurons. The second of these is the main topic of this thesis and will be discussed in the Results and Discussion section.

3.3.3 Transdifferentiation – the direct conversion of starter cell to neuron

Many studies now show that it is possible to force cells to turn from one cell type into another by overexpressing select transcription factors in them (*176–178*, *83*, *179*). In the brain, this has been successful using astrocytes or NG2 cells as starter cells. The transcription factors used are selected for their important roles in instructing neuronal identity during normal brain development. When these genes are artificially overexpressed in adult cells, they work by superimposing a neuronal transcription factor network onto that of the starting cell, leading to direct transdifferentiation within a few days. For example, the proneural transcription factor Ascl1 is often included for its important ability to make chromatin more accessible and allow other transcription factors to bind DNA (*180*). Such other transcription factors often specify a broad neuronal identity and include Brn2, Myt1l, Zfp238, Sox2, Neurog2, Neurod1, Pax6 and Dlx2 (*178*, *83*, *181*, *182*, *81*, *183*). Interestingly, although reprogramming has most often been induced by forced overexpression of transcription factors, a number of studies now

show that *in vitro* reprogramming is also possible by using pharmaceutical compounds alone (*90*, *89*, *184*).

In one study, single-cell RNA sequencing was used to study the transcriptional changes that occur as fibroblasts are converted to neurons using Brn2, Ascl1 and Myt1l *in vitro* (*185*). As transdifferentiation was initiated, the authors found, the starting cell's transcriptional profile was gradually lost, and at the same time, that of the target cell was gradually acquired. Interestingly, genes associated with neural stem cells were transiently expressed halfway through the process. Even so, direct transdifferentiation does not proceed along the same steps as normal neuronal differentiation does (*174*, *186*). Importantly, cells do not pass through a bona fide stem cell state, which includes proliferation to generate transit-amplifying cells. Indeed, direct transdifferentiation does not require cell division at all (*187*). This means that for every neuron that is generated, one starter cell is consumed. It is possible that neuronal transdifferentiation on a massive, therapeutic scale may thus significantly impact the ratio of neurons to glia in the brain, which is roughly 4:1 in the cortex, 1:4 in the cerebellum, and 11:1 in the basal ganglia, diencephalon and brainstem (*63*). Whether this would have any functional consequences is not known, but the ratio of glia to neurons is probably important for proper function of neural circuits: Disturbances in this ratio are seen in many neurodevelopmental disorders (*188*). It is, however, equally possible that remaining glial cells would step in and replenish their own numbers through cell division. In mice, this has been conceptually demonstrated for NG2 cells, which proliferate to re-establish correct numbers if some of them are lost (*66*). Astrocytes, too, can divide to generate more astrocytes after brain injury (*65*).

The fact that there is no expansion in cell numbers during the process of direct transdifferentiation suggests that this method may not be suitable for generating a large number of neurons. This prompts the question of how many new neurons are actually needed for there to be a significant improvement in brain function. The transplantation studies mentioned above showed that behavioral improvements could be detected after transplantation of only a few thousand neurons (*71*). Importantly, such positive effects have also been seen when neurons were generated through transdifferentiation of endogenous cells. In one study, the generation of a few hundred dopaminergic neurons, at most, led to a small but statistically significant behavioral improvement in a mouse model of Parkinson's disease (*189*)

These advances are promising. Nonetheless, an attractive alternative to direct reprogramming might be to make each starter cell generate many neurons instead of just one. As we have seen, the tissues and organs with the highest demand for cell replacement tend to employ stem cells, a setup that allows each starter cell to generate a large number of progeny via highly proliferative transit-amplifying cells. This is also the mode by which neural stem cells generate neurons in the subventricular zone and dentate gyrus. Considering the close relationship between neural stem cells and astrocytes, an exciting possibility is therefore whether astrocytes can be converted into neural stem cells.

4 CONCLUDING REMARKS

Having surveyed the reparative capacity of many tissues and organs, we can now take a more holistic view and place the regenerative capacity of the central nervous system in a bigger context. It is clear that injury repair in the brain and spinal cord suffers from the same limitations as those seen in other organs: a tendency for scar formation, imperfect cell replacement, and a poor capacity for tissue patterning. However, in the central nervous system, these limitations have some very special consequences. For example, a scar in the brain does not substitute for any normal brain function, the way it does in structural tissues like the skin. Cell loss, too, has particularly devastating consequences in the brain. This is because each brain region is more or less uniquely responsible for a particular body function. Therefore, even small lesions may lead to complete loss of certain brain functions. In contrast, the function of many other organs is distributed evenly across the entire organ. In organs like the lungs and the intestine, damage tends to cause a gradual worsening of overall tissue function. Finally, the central nervous system's function is almost entirely determined by the complex circuitry of its neurons. This places great demand on the capacity of the brain to establish an appropriate tissue patterning environment after injury. If the healing brain fails to provide the patterning signals required for axons to reach their appropriate targets, tissue function will not be properly restored. This is in contrast to an organ like the liver, which does not require perfect tissue patterning to become functionally equivalent to what it was before an injury.

The brain does generate new neurons in certain regions throughout adulthood, and it does so through the use of specialized stem cells. Conspicuously enough, in other parts of the body adult stem cells are a hallmark feature of organs that require very rapid cell turnover for normal tissue function. Clearly then, the fact that the neurogenic regions are so obviously optimized for high neuronal output must say something about the evolutionary pressures that have shaped adult neurogenesis in our ancestors, and possibly also about the importance of this phenomenon in the modern human brain. Yet, this sleek neurogenic machinery has evolved right in the middle of an organ that is otherwise almost totally devoid of neuronal replacement – a seemingly paradoxical contrast.

Throughout the body, organs with low to medium levels of cell turnover often rely on the duplication of pre-existing differentiated cells for tissue homeostasis; examples include such diverse cell types as hepatocytes, microglia and most epithelial cells (*3*, *4*, *6*, *7*). This mechanism would seem to be appropriate also for the replacement of neurons in the brain parenchyma, as these normally require very little cell replacement. However, mature neurons do not replicate themselves. In fact, if artificially stimulated to divide, neurons would rather undergo apoptosis than enter the cell cycle (*190*). Thus, the fact that the brain is unable to use this simple mechanism to replicate its neurons could be an underlying factor for its poor reparative capacity.

Outside the brain, one additional mechanism exists for replacing cells when the normal modes of cell replacement are not enough: facultative stem cells. As described previously, severe injuries can lead to the recruitment of stem cells which do not normally act as stem cells. In some organs, these cells appear to do little else than wait to be called into action – for example, satellite cells in skeletal muscle or +4 cells in intestinal crypts. In other cases, they serve specialized roles unrelated to their latent stem cell capacity – for example, club cells in the lungs, or tubular epithelial cells in the kidneys. Turning to the brain, the question has remained whether any facultative stem cells exist whose neurogenic capacity has not yet been discovered. In the peripheral nervous system, the astrocyte-like satellite glial cells fulfill this role, as discussed previously. The fact that astrocytes are conspicuously similar to neural stem cells, and the observation that they can display neurogenic properties if isolated and cultured *in vitro*, has suggested that a closer study of this cell type's neurogenic capacity *in vivo* may be warranted.

5 METHODS

5.1 CELL FATE MAPPING

Adult stem cells can often not be recognized in histological tissue sections because stem cells do not have a characteristic morphology that sets them apart from non-stem cells. Instead, identification of stem cells relies on the experimental labeling of cells at one time point and, at a later time point, analysis of the labeled cells to see what they have done. One way of doing this is to treat animals with a compound called bromodeoxyuridine (BrdU), a DNA nucleotide analog containing a bromine atom in place of a CH3 group. When any cell in the body divides, it synthesizes new DNA and will randomly incorporate the blood-borne BrdU into its genome. Later, tissue sections can be analyzed using fluorescently labeled antibodies against BrdU, and in this way it is possible to see what cells had become that divided at the time of BrdU administration. This technique and variations of it are tremendously useful in stem cell biology. For example, the first generation of nucleotide analogs were based on radioactive thymidine. It was these that enabled the first evidence of adult-born neurons in the rat dentate gyrus (*95*, *96*).

However, BrdU is incorporated into any cell that divides. Therefore, if a $BrdU^+$ cell is spotted in the tissue, it is not possible to deduce its origin - whether it was generated through the mere duplication of an identical cell or whether a stem cell gave rise to it. For this reason, a different technique has become even more useful - genetic fate mapping (also called lineage tracing). This technique relies on the use of genetically modified animals, often mice, that carry a specific genetic construct. Here, the DNA-cutting enzyme Cre is expressed under the control of a cell type-specific promoter. In Papers I and II, Cre was expressed under the control of the astrocyte-specific Connexin-30 promoter, and thus, Cre is expressed only in astrocytes. In a further twist, Cre is fused to the estrogen receptor (ER), which means that the CreER fusion protein cannot enter the nucleus unless estrogen or an analog like tamoxifen is present. Once mice are treated with tamoxifen, CreER enters the cell nucleus and makes a cut in another transgene, the result of which is the heritable expression of a fluorescent reporter gene such as yellow fluorescent protein (YFP) or tdTomato. In effect, tamoxifen administration makes astrocytes fluorescent, and any cell type the astrocytes might give rise to will also be fluorescent. This technique is enabling the identification of stem and progenitor cells throughout the body of experimental animals. It does, however, require the use of transgenic animals, the generation and maintenance of which is very time consuming. Each such mouse strain enables the tracing of only one or a few cell types, making a detailed mapping of the developmental potential of every cell type in the body very work-intensive. In addition, because not all cell types have unique marker genes that set them apart from other neighboring cells, transgenic mice do not exist for every cell type in the body.

5.2 RADIOCARBON DATING TO ESTIMATE CELL TURNOVER

One significant limitation with BrdU and genetic fate mapping is that they are not routinely applicable to humans for ethical reasons. Therefore, a technique has been developed in the Frisén lab that uses ^{14}C dating to birth date human cells in postmortem samples. The atmospheric levels of ^{14}C have been stable for a long time but rose drastically as a result of above-ground nuclear bomb tests between the 1950s and early 1960s (*191*, *192*). Following the partial nuclear test ban treaty in 1963, the atmospheric ^{14}C levels started decreasing exponentially, not due to radioactive decay (^{14}C has a half-life of 5730 years) but due to equilibration with the oceans and the biosphere (*193*). Plants take up atmospheric ^{14}C through photosynthesis, and humans take up ^{14}C by eating plants or animals that live off plants. Therefore, the ^{14}C concentration in our body reflects that in the atmosphere at any time (*194*, *195*). When a cell in our body divides, it synthesizes new DNA, which consists of 30% carbon. Its genomic ^{14}C concentration therefore corresponds to that in the atmosphere at the time the cell divided. Because DNA is a very stable molecule, every cell in effect has its birth date stamped into its DNA, written in ^{14}C. By isolating the DNA from a population of cells and measuring the ^{14}C concentration using accelerator mass spectroscopy, the average age of that cell population can be measured. This method has been used extensively by the Frisén lab to assess the turnover dynamics of various cell populations in the adult body (*6*, *101*, *196*, *197*). In Paper III, we applied this technique to address whether stroke leads to increased neurogenesis in the striatum of humans.

This method is powerful because it enables the retrospective birth dating of cells in humans without requiring pre-treatment of any tracer molecule like BrdU. We are all, in principle, labeling ourselves all the time through the food we eat. However, experimental protocols are very time-consuming and work-intensive, as each investigated cell type requires material from many subjects, which can take months or years to collect. In addition, the method requires cell nuclei to be isolated by labeling them with fluorescent antibodies against unique nuclear markers. Such markers do not exist for all cell types. Furthermore, the limited sensitivity of accelerator mass spectrometry requires millions of nuclei to be extracted from each subject, which may not always be possible if the cell population of interest is small.

5.3 SINGLE-CELL RNA SEQUENCING

A cell's identity and behavior is governed by the genes it expresses. Sequencing a cell's transcriptome thus gives a snapshot picture of the cell's current state. The recent development of "next-generation" sequencing has enabled the rapid emergence of single-cell sequencing as a field that is now evolving at a high pace (*198*). By analyzing cells on the single-cell level, cell heterogeneity can be dissected in ways that are not possible when studying cells in bulk. For example, a collection of actively proliferating stem cells and their progeny will contain cells in all stages of maturation. Only through single-cell analyses can this heterogeneity be resolved. Therefore, single-cell RNA sequencing is now being used to generate an extensive

catalog of the cell types and developmental stages that exist in the body. In Paper II, single-cell RNA sequencing was used to analyze astrocytes undergoing neurogenesis *in vivo*, with two aims in mind. The first aim was to computationally reconstruct the differentiation trajectory of striatal neurogenic astrocytes, to understand this biological process in more detail. The second aim was to compare gene expression between astrocytes that underwent neurogenesis with those that did not. Differentially expressed genes highlighted a number of signaling pathways whose activity level might confer neurogenic ability on astrocytes. We used this information to guide further in-vivo interventions whose aim was to stimulate neurogenic lineage progression in non-neurogenic astrocytes. Applying single-cell RNA sequencing analysis in this way has not been extensively used before.

Today, a handful of techniques exist for preparing RNA libraries for sequencing. In Paper II, we used SmartSeq2, a protocol whose strength is that it enables the detection of full-length transcripts, but has the drawback that absolute transcript counts based on unique molecular identifiers (UMIs) cannot be used (*199*). Computational tools abound for computationally classifying cell types in a heterogeneous population or cells along different stages of a differentiation process. These tools are available and are relatively easy to apply in programming languages like R thanks to the existence of many pre-made packages.

6 PRESENT INVESTIGATION

6.1 AIMS

Paper I – To investigate whether astrocytes in the adult mouse brain can generate neurons in response to stroke, and if so, what the molecular mechanisms are that govern this process

Paper II – Aim 1: To use single-cell RNA sequencing to investigate the transcriptional mechanisms underlying astrocyte neurogenesis. Aim 2: To compare gene expression between neurogenic and non-neurogenic astrocytes, using this information to guide interventions that may stimulate astrocytes to undergo neurogenesis in otherwise non-neurogenic regions.

Paper III – To investigate whether stroke-induced neurogenesis occurs in the human striatum

Paper IV – To develop *spatial transcriptomics*, a method for performing RNA sequencing on intact tissue sections with retained spatial information

6.2 PAPER I

6.2.1 Summary of results

The similarity between neural stem cells and astrocytes (Section 3.2) prompted the question of whether astrocytes can generate neurons *in vivo* under some circumstances. This question was addressed in Paper I.

Using transgenic reporter mice expressing CreER under the control of an astrocyte-specific Connexin-30 (Cx30) promoter, we could label astrocytes with heritable YFP expression in the healthy brain and follow their fate after stroke. Indeed, from two weeks after stroke and onwards, we observed YFP^+ transit-amplifying cells and neuroblasts in the striatum. Many of these cells were found in proliferating clusters, suggesting that they were being produced on site. At a later time point, seven weeks after stroke, we even observed that some of these cells had developed into mature interneurons. This showed that the Cx30-$CreER^+$ cells that were labeled before the stroke could generate neurons.

However, the Cx30-CreER mice labeled not only astrocytes, but also neural stem cells in the subventricular zone. Subventricular zone-derived neuroblasts can migrate into the stroke-injured striatum (*155*, *156*). We could therefore not exclude that the YFP^+ neuroblasts and neurons we observed in the striatum had migrated from the subventricular zone. For this reason, we used a different method to follow the fate only of striatal astrocytes. We injected an astrocyte-specific adenovirus, carrying the Cre gene, into the striatum. This allowed us to activate YFP expression only in striatal astrocytes and not in subventricular zone cells. When these mice were analyzed after stroke, we found that some striatal transit-amplifying cells and neuroblasts were YFP^+, meaning that they had indeed been generated by the striatal astrocytes that were labeled prior to stroke.

We asked what the molecular mechanism is that regulates stroke-induced astrocyte neurogenesis. The Notch signaling pathway is a known regulator of neurogenesis by neural stem cells. For example, if the Notch-mediating transcription factor *Rbpj* is deleted in subventricular zone stem cells, they all undergo premature neurogenesis and are themselves consumed by this process (*200*). We asked whether astrocyte neurogenesis, too, is regulated by Notch. For this, we used a variant of the Cx30-CreER mouse that carried an inducible form of the activated Notch1 receptor (NICD). In effect, astrocytes in these mice had constantly active Notch signaling. We reasoned that if Notch downregulation is the mechanism by which stroke normally triggers astrocyte neurogenesis, astrocytes in this mouse should be unable to undergo neurogenesis. That was indeed what we found. After stroke, none of the NICD-expressing astrocytes had generated transit-amplifying cells or neuroblasts. This showed that Notch downregulation normally is necessary for astrocyte neurogenesis after stroke.

If astrocytes require Notch downregulation to initiate their neurogenic program, we finally asked whether astrocyte neurogenesis could be activated even in the absence of stroke by artificially inactivating the Notch pathway. For this, we used transgenic Cx30-CreER mice

carrying inducible null mutations in *Rbpj*, the crucial Notch-mediating gene. When *Rbpj* was deleted in astrocytes throughout the brain of healthy mice, we found that they generated transit-amplifying cells, neuroblasts and neurons, both in the striatum and in a narrow region of the midline cortex, even in the absence of stroke. To exclude that these cells had been generated by migrating subventricular zone cells, we repeated the experiment but this time deleted *Rbpj* exclusively in striatal astrocytes using the Cre-expressing adenovirus. Indeed, this experiment confirmed the previous one: Inactivation of the Notch pathway in striatal astrocytes is sufficient to trigger entry into a neurogenic program, whereby astrocytes initiate transit-amplifying divisions and generate neuroblasts, some of which mature into interneurons.

6.2.2 Discussion (Paper I)

These results are highly interesting because they demonstrate for the first time that astrocytes can generate neurons in the living brain (Figure 8). In effect, these results serve to blur the already diffuse boundary between astrocytes and neural stem cells (see Figure 7). Shortly after these results were published, another research group published a similar finding, that astrocytes in the adult mouse striatum can generate neuroblasts in response to an excitotoxic lesion (*113*). Together, these results indicate that some astrocytes in the mouse brain show the classic hallmarks of facultative stem cells (Section 2.3), namely an extended state of quiescence followed by injury-induced activation of stem cell properties.

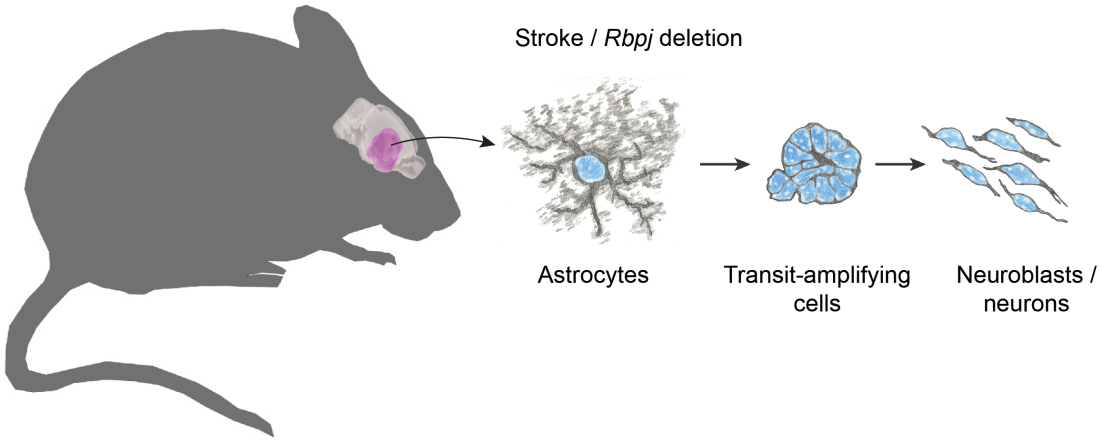

Figure 8. Astrocytes in the striatum undergo neurogenesis in response to stroke or *Rbpj* deletion. Paper I shows that astrocytes in the adult mouse striatum activate a neurogenic program in response to experimental stroke or *Rbpj* deletion. They generate transit-amplifying cells that divide four to five times, generating ~40 neuroblasts per starting astrocyte. Some of these neuroblasts mature into neurons.

Interestingly, activation of astrocyte neurogenesis in the healthy mouse striatum by genetic manipulation was shown in yet another study (*201*). Here, the overexpression of Sox2 made astrocytes initiate rounds of transit-amplifying divisions and generate neuroblasts, in a process that looked identical to what we observed after *Rbpj* deletion. It is interesting to speculate that many possible ways may exist to provoke the molecular programs that govern astrocyte neurogenesis. Mechanistically, this might be explained by the stabilization or destabilization of cross-repressing transcriptional programs responsible for governing competing cell identities (*9*).

The results presented in Paper I introduce the tantalizing possibility that it might be possible to recruit astrocytes throughout the brain as an abundant source of new neurons. However, following the observations presented here, two questions presented themselves: Why could astrocyte neurogenesis only be provoked in the striatum and midline cortex, and not in other regions of the central nervous system? And do humans, too, have stroke-induced neurogenesis in the striatum?

6.3 PAPER II

6.3.1 Summary of results

In Paper I, we had found that *Rbpj* deletion activates neurogenesis by astrocytes in the striatum and midline cortex. However, in no other regions did astrocytes undergo neurogenesis, despite having lost the *Rbpj* gene. Single-cell RNA sequencing is a powerful method for understanding cells on a transcriptional level and it enables the reconstruction of cell differentiation trajectories using computational tools. We asked whether we could use single-cell RNA sequencing to characterize the transcriptional programs underlying astrocyte neurogenesis in the striatum, and understand why astrocytes in other regions are unable to undergo neurogenesis.

Our single-cell RNA sequencing analyses showed that astrocyte neurogenesis happens through the same general steps as that by adult neural stem cells. In particular, genes associated with gene expression and cellular metabolism are upregulated early as the neurogenic program is activated. Astrocytes then undergo rounds of transit-amplifying divisions to generate neuroblasts. A direct comparison with a previously published dataset of subventricular zone stem cells and their progeny (*146*) revealed that astrocytes are located "upstream" of neural stem cells on one single differentiation trajectory, as if representing a highly quiescent form of neural stem cells (Figure 9).

Our experiments from Paper I had shown that even though striatal astrocytes can undergo neurogenesis after *Rbpj* deletion, not all of them do so. Our RNA sequencing data corroborated these findings, suggesting that many striatal astrocytes halted their neuronal lineage progression immediately prior to cell cycle entry. By stimulating cell division of striatal *Rbpj*-deficient astrocytes using the growth factor EGF or experimental stroke, we

could greatly increase the amount of astrocytes that entered transit-amplifying divisions, demonstrating the importance of mitogenic stimuli for proper neurogenic lineage progression.

Next, we asked why astrocytes outside the striatum and midline cortex could not complete neurogenesis after *Rbpj* deletion. Our RNA sequencing data showed that even in the non-neurogenic somatosensory cortex, *Rbpj*-deficient astrocytes did in fact initiate neurogenesis but that they, too, halted their lineage progression immediately prior to cell cycle entry. We found that pre-division astrocytes in the somatosensory cortex differed from those in the striatum mainly with regard to genes involved in proliferation-regulating pathways, like MAPK/ERK, PI3K/AKT and Wnt. These results suggested that strong enough mitogenic stimuli might be able to push *Rbpj*-deficient astrocytes into transit-amplifying divisions and neurogenesis in the somatosensory cortex and, perhaps, other parts of the central nervous system. Identifying such mitogenic stimuli will be an important goal for future studies.

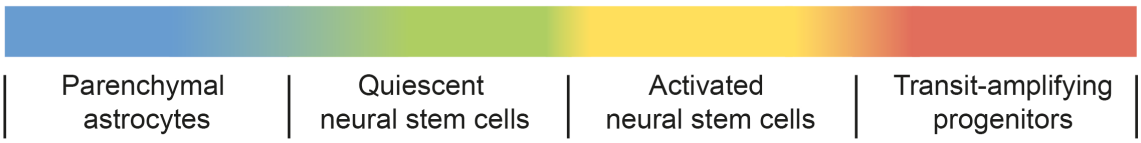

Figure 9. Astrocytes are located upstream of neural stem cells on a one-dimensional differentiation trajectory. Results from Paper II indicate that astrocytes represent a highly quiescent form of neural stem cells, illustrated in this schematic representation of a one-dimensional differentiation path. After stroke or *Rbpj* deletion, they fall in line with the same neurogenic trajectory as that of adult neural stem cells. Image credit: Mattias Karlén

6.3.2 Discussion (Paper II)

The findings presented in this study are interesting for a number of reasons. First, they show that the neurogenic programs of astrocytes and neural stem cells are very similar on the transcriptional level. This indicates that it may be meaningful to think of astrocytes as highly quiescent neural stem cells. Secondly, these results show that Notch inactivation is sufficient to initiate the neurogenic program in astrocytes even outside the striatum and midline cortex. Nonetheless, an additional push by mitogenic signals might be needed to force these primed astrocytes into transit-amplifying divisions. In other words, the Notch downregulation that occurs naturally as a reaction to a nearby stroke may serve to place astrocytes in a "primed" state, in which subsequent mitogenic stimuli activate actual neurogenesis. Such injury-induced priming has been described also for the stem cells of skeletal muscle – the satellite cells. These cells enter a primed state as a reaction to a distant injury, in which they are poised for cell cycle entry (*202*). This type of priming is useful because the first cell division after quiescent stem cell activation takes a long time to complete (*203*). Thus, initial priming

followed by a requirement for mitogens might represent a general principle of facultative stem cell activation. Interestingly, even the molecular mechanisms may be similar between astrocytes and as satellite cells, as the latter cell type's re-entry into quiescence depends on a reactivation of Notch signaling (*204*).

An additional mechanistic insight into the molecular program underlying astrocyte neurogenesis concerns the role of reactive gliosis. Previous studies have shown that astrocytes can be isolated from the injured mouse brain and generate neurons *in vitro*, as discussed in Section 3.2 (*152–154*). The requirement for a preceding injury in some studies has led to the conclusion that reactive gliosis (Section 3.2) is a prerequisite for the in-vitro neurogenic properties of astrocytes. The results from both Paper I and Paper II show, however, that astrocyte neurogenesis does not fundamentally require reactive gliosis. After *Rbpj* deletion in healthy mice, astrocytes in the striatum and midline cortex underwent neurogenesis without any signs of reactive gliosis (Paper I). Even after stroke, Ascl1-expressing astrocytes existed that did not show signs of reactive gliosis. Conversely, only a minority of reactive astrocytes expressed Ascl1. This demonstrates that the transcriptional programs that govern astrocyte neurogenesis and reactive gliosis are not necessarily coupled, though they are mutually compatible. However, reactive gliosis may be important because it primes cells for division. In Paper II, our results clearly highlight the importance of mitogenic stimuli for neurogenic astrocytes to enter transit-amplifying divisions. Because severe brain injury leads to astrocyte proliferation (*65*), these results suggest that the mechanism by which reactive gliosis facilitates astrocyte neurogenesis is by making astrocytes more prone to enter the cell cycle.

One main insight from Paper II is that astrocytes from the non-neurogenic somatosensory cortex, and perhaps throughout the central nervous system, have an intrinsic capacity to generate neurons but that not all of them display this capacity *in vivo*. Our analysis highlighted the importance of regional differences in the extracellular environment for influencing astrocyte neurogenesis. However, it should not be ruled out that cell-intrinsic differences between astrocytes exist as well. Astrocytes are variable in both form and function. For example, one of the clearest differences in astrocyte morphology can be seen between those in the gray and white matter. Even so, apart from such morphological variety, the extent and origin of astrocyte heterogeneity is not well known. But emerging evidence is suggesting that astrocytes may actually be surprisingly homogeneous on the transcriptome level, at least compared to neurons. Such evidence comes primarily from RNA sequencing studies. In one study, where cells from the mouse cortex and hippocampus were analyzed using single-cell RNA sequencing, only 2 astrocyte subtypes were detected, compared to 29 subtypes of neurons (*205*). A similar study found that astrocytes in the mouse striatum did not fall into clear subgroups at all, but were distributed along a diffuse continuum of subtle transcriptional differences (*206*).

How such differences come about is not well known. One possibility is that they were imbued into astrocyte progenitors already during early nervous system development. In the

developing neural tube, dorsoventral patterning generates what will later become distinct radial glial domains in the ventricular zone (*207*). Later during development, the radial glia-derived astrocytes migrate radially outwards from the ventricular zone to take up regionally distinct positions in the brain, where they remain for life (*208*). Because the distribution of adult astrocytes reflects the radial glial domains in the early neural tube, it is theoretically possible that some regional properties were inherited from the earliest tissue patterning events. However, the extent to which this is actually true is not known.

An alternative hypothesis, for which there is evidence, posits that regional differences between astrocytes instead arise as adaptations to local environmental signals. One study showed that some properties of astrocytes in the cerebellar cortex are influenced by signals from surrounding neurons – in particular secreted Sonic hedgehog (*209*). This suggests that many other astrocyte properties, too, may be dictated by environmental signals. For example, the intrinsic neurogenic property observed in some astrocytes could in principle be subject to such environmental regulation. Whether this is actually the case is not known, but this question is the main topic of this thesis.

Taken together, current evidence suggests that astrocytes are much less diverse than neurons and that the differences between them are subtle and arise as local adaptations to the needs of surrounding neurons. Yet, it should not be ruled out that some form of astrocyte heterogeneity remains from differences in astrocyte progenitors during brain development – perhaps also differences in neurogenic capacity.

6.4 PAPER III

6.4.1 Summary of results

A well established fact is that stroke-induced neurogenesis takes place in the striatum of rodents (*123*). Many of the neuroblasts that appear in the striatum have migrated from the nearby subventricular zone (*155*, *156*); others have been generated by local striatal astrocytes, as we show in Paper I. Whether anything similar happens in the human stroke-injured brain is not known, but the answer is important: Stroke often leads to lifelong handicaps, but it is important to know whether recovery is poor because there is no neuronal replacement, or whether recovery is poor *despite* neuronal replacement. The answer to this question will guide our thinking about future strategies to improve functional recovery with cell therapy.

Radiocarbon dating can be used to estimate the turnover dynamics of a cell population (*196*). In a previous study from our group, radiocarbon dating of cortical neurons from stroke survivors showed that there is no stroke-induced neurogenesis in this brain region (*133*). However, the striatum may be different, as there is adult neurogenesis here already in the healthy human brain (*109*). In Paper III, we used radiocarbon dating to assess whether stroke leads to an increase in striatal neurogenesis in humans.

Results from the radiocarbon dating analysis showed that there is indeed a higher proportion of young neurons in the stroke-injured striatum, compared to the non-injured control striatum of the same patients. This is suggestive of neurogenesis but is also compatible with selective death of the oldest neurons, a scenario possible only in brain regions with lifelong adult neurogenesis, like the striatum. Mathematical modeling indicated that even if old neurons would be 10% more likely to die than young ones, the high neuronal ^{14}C values of some subjects were best explained by stroke-induced neurogenesis.

In the healthy human striatum, neuronal turnover is restricted to interneurons (*109*). In subsequent histology analyses, we therefore quantified the proportion of interneurons in the stroke-injured striatum. In addition, we assessed the proportion of neurons containing the age pigment lipofuscin, as a proxy for cell age. Both of these analyses indicated that there is an increased proportion of young neurons in the stroke-injured striatum. However, neither analysis could discriminate between neurogenesis and selective death of old neurons.

6.4.2 Discussion (Paper III)

Our results show that stroke leads to an increased proportion of young neurons in the stroke-injured human striatum. However, these results could in theory be explained by one of two different scenarios: stroke-induced neurogenesis or selective death of old neurons. This issue might be resolved by analyzing more subjects that died very shortly after their stroke. This is because any selective neuronal death would likely occur immediately during stroke, whereas a population of newborn neurons would take months or years to accumulate. Therefore, subjects in which any putative selective neuronal death had occurred, but not much neurogenesis, would allow an estimation of the extent of selective neuronal death. Interestingly, one such subject already existed in our dataset, and their ^{14}C values were tantalizing: This individual was the only one who did not show an increased proportion of young neurons in their stroke-injured striatum. If anything, this subject showed an increased proportion of *old* neurons, suggesting selective death of *young* neurons. Such a selective death of young neurons would fit well with a previous radiocarbon dating study that showed that Huntington's disease causes selective death of adult-born neurons rather than the oldest neurons (*109*). In the current study, the results from this one subject therefore suggest that any putative selective death of old neurons is not great enough to cause a detectable increase in neuronal ^{14}C concentration. Instead, the increased ^{14}C concentration in the stroke-injured striatum observed in all other subjects could therefore be best explained by neurogenesis. It will be of great importance to analyze more such subjects.

One conclusion we can draw with certainty from our data is that the proportion of young interneurons is increased in the stroke-injured striatum compared to the healthy striatum. It is not known what the functional consequence of this is, but it is likely to be of some relevance as interneurons and projection neurons have different roles in sculpting neuronal circuits.

Recovery is often poor in stroke patients, and it is important to know whether this is due to a total lack of neuronal replacement or despite some neuronal replacement. The reason this is important is that such knowledge may guide future strategies to improve stroke recovery through artificially induced neuronal replacement. If there is already some stroke-induced neurogenesis in the brain, this means that the brain has mechanisms in place to generate new neurons and that this process might be boosted. On the other hand, it would mean that stroke recovery is poor *despite* stroke-induced neurogenesis, which might limit the usefulness of neuronal replacement as a therapeutic strategy. If it turns out that there is no stroke-induced neurogenesis at all, it is harder to envision how to achieve therapeutic neuronal replacement, as there would be no naturally occurring process that could be boosted. On the other hand, if there is no neuronal replacement taking place naturally, chances are that adding just a few artificially may have a large beneficial impact.

What might be the origin of any stroke-induced striatal neurons? As discussed extensively in this thesis, striatal astrocytes of mice can activate a neurogenic program in response to stroke. Could the same be true of human striatal astrocytes? Much of what we know about astrocytes comes from studies in rodents, even though it is known that human astrocytes look very different and are more diverse than those in mice and rats. One study that compared the genomes of brain cells in humans and mice suggested that it was in fact among glial transcripts that the biggest species differences existed between the two species (*210*). Human astrocytes occupy a volume 17 times as large as that of mouse astrocytes, project ten times as many $GFAP^+$ filaments and have more diverse shapes (*211*). They might even be superior to mouse astrocytes in the way they regulate neuronal signaling. Incredibly, one research group transplanted human glial progenitor cells to the developing mouse brain and showed that the adult mice, now having human astrocytes in their brains, displayed increased cognitive capacity (*212*).

Whether human and rodent astrocytes differ in their intrinsic capacity to generate neurons is not known. Some studies have reported that cells can be isolated from the human cortex and form neurons *in vitro* if stimulated with growth factors (*213*, *214*), though the identity of these neurogenic cells was not shown. Other studies, in contrast, report that no such cells can be isolated from the human brain (*215*, *216*). These opposing results might possibly be explained by different culturing protocols or postmortem time. In the near future, one of the most pressing questions in the field will be to characterize the neurogenic potential of human astrocytes.

What would be the mechanism by which new neurons benefit the injured brain? The most obvious answer is that each new neuron would take the exact place of one dead neuron, such that all neuronal subtypes and their connections were restored. Although it is possible to imagine a scenario where we are able to regenerate all neuronal subtypes at correct proportions, perfect neuronal replacement will likely be extremely difficult to achieve. One reason for this was discussed above, namely that the tissue patterning signals that guided neural connectivity during development are no longer present in the adult. Therefore, new

neurons could probably not re-establish long-range neuronal connections. Even salamanders, the masters of regeneration among the vertebrates, cannot re-establish long-range neural connections after injury (*54*), probably for a lack of patterning signals. Another reason why perfect neuronal replacement is not likely to be achieved in the near future is that brain injury leads to massive scarring and tissue remodeling. Only adding neurons will not restore a lesion site to its original condition. Rather, biological engineering on a scale and precision only dreamed of today would likely be needed to revert an injury-remodeled tissue to its former state. For these reasons, any beneficial effect of new neurons would likely be of a different kind.

One scenario is that new neurons act as relays that reconnect two spared neurons that used to be directly connected to one another. This has been demonstrated in one study of spinal cord injury in rats. Here, neural stem cells grafted to the lesion site developed into neurons that received connections from endogenous descending motor neurons and in turn projected their own axons to distal targets (*217*).

A second scenario is that new neurons are used only as a source for the neurotransmitters they secrete. This is the case with strategies to replace dopamine-producing neurons in Parkinson's disease patients. In experimental and clinical studies, dopaminergic neurons have been transplanted to the striatum, which is the region where dopamine is needed, but not where dopaminergic neurons are normally located. This seems not to matter, however, as transplanted neurons can secrete dopamine and re-establish striatal circuit function (*72*).

A third scenario is to generate interneurons that tune and modulate spared neuronal circuits. Interneurons make up only 10-15% of all neurons in the rodent brain (*218*), but despite their scarcity, they have a huge impact on neuronal circuit output. This is because they can modulate and coordinate the firing pattern of surrounding neurons (*219*). Furthermore, their impact on a circuit is not only determined by their numbers; rather, individual interneurons can tune the strength of their inhibitory signals onto surrounding neurons (*220*). This suggests that individual interneurons change their output strength to match the need of their local circuit, potentially giving each interneuron very large influence over the circuitry. In turn, this suggests that functionally significant effects on brain recovery may be achieved if only a small amount of new interneurons could be generated. Indeed, as described below, the small number of neurons generated in response to stroke in the rodent striatum are mostly interneurons (*112*, *122*), suggesting that interneuron generation may be the actual "strategy" used by the injured brain to sculpt spared neuronal circuits.

As a side note, it is interesting to speculate whether cognitive performance of the healthy brain, too, would be improved by adding more neurons. After all, cognitive capacity appears to be positively correlated with neuronal density: Comparisons between species show that primates, who have comparatively high cognitive capacity, have brains particularly densely packed with neurons. For example, the brain of the capuchin monkey contains more than twice as many neurons as the similar-sized brain of the capybara, a large rodent (*221*). One idea, then, is that neuronal density is causally correlated with cognitive capacity and that we

could in effect become "smarter" by increasing our neuronal density even further. This may be the case; the answer is not known. However, species comparisons within primates show that the human brain's neuronal density is not higher than that of other primates. Our brains are simply bigger than those of other primates and therefore have more neurons (*221*). To understand whether the healthy brain's cognitive capacity could be improved with greater numbers of neurons, it may also be instructive to study the developing brain, where neuronal numbers are precisely tuned. During brain development, more neurons are generated than are needed, but in early postnatal life roughly half of all neurons die, through mechanisms that are not fully understood (*220*, *222*). In one study, immature interneurons were transplanted to the postnatal mouse brain in an attempt to increase the number of neurons (*220*). Though many of these transplanted neurons died, it was possible to increase the total number of interneurons in the brain by up to 35%. Interestingly, even though the role of interneurons is to inhibit surrounding projection neurons, there was no increase in neuronal inhibition as a result of the more abundant interneurons. This means that individual interneurons tuned the strength of their inhibitory signals to compensate for their higher numbers, such that the performance of the circuit was optimized. By analogy, then, these results suggest that adding more neurons to the healthy adult brain would not lead to an improved computing power of neural circuits. Rather, homeostatic mechanisms would act to maintain circuit performance in its ground state.

6.5 PAPER IV

6.5.1 Summary of results

Analysis of gene expression in histological tissue sections is a cornerstone of biomedical research. With methods like in situ hybridization, it is possible to study how gene expression differs between regions in a tissue. With this method, however, analysis is usually limited to one single gene at a time (though newer variants allow the detection of hundreds of genes at a time (*223*)). In contrast, recently developed methods like single-cell RNA sequencing enable the detection of practically the entire transcriptome in a single experiment. Here, however, information about each cell's position in the tissue is lost. There has been a lack of methods for analyzing the entire transcriptome and simultaneously retain information about the spatial expression pattern of genes.

In Paper IV, we developed *spatial transcriptomics*, a technology that addresses this issue. Here, an intact tissue section is placed onto a chip and treated such that cellular RNA leaks out to the surface of the chip. The chip's surface is covered with DNA oligonucleotide probes. These bind to the RNA and additionally contain barcode sequences unique to each probe's position on the chip. By sequencing the probes with the captured RNA, transcriptional information is retrieved and each transcript's spatial information can be reconstructed.

We first applied this method to the mouse olfactory bulb, a structure whose distinct spatial features make it suitable for proof-of-concept implementation. Initial tests showed that a permeabilized tissue section releases RNA onto the chip with minimal sideways leakage, the hybridized RNA generating a near-perfect spatial representation of the tissue's cells on the chip. After sequencing, we measured the sensitivity of transcript detection to be on the lower end of the sensitivity span reported for single-cell RNA sequencing. Compared to the gold standard (single-molecule fluorescent in situ hybridization [smFISH]), spatial transcriptomics detected 0.069 times as many transcripts.

Each region in the olfactory bulb generated sequencing data that matched the known cell types in that region. An unbiased dimensionality reduction analysis (tSNE) of all sequencing data was able to distinguish each region computationally. These results showed that spatial transcriptomics works and can be used to ask biologically relevant questions about spatial differences in gene expression.

Finally, we asked whether spatial transcriptomics could generate clinically valuable information in cancer diagnostics. We therefore analyzed a human breast cancer biopsy that contained regions of invasive ductal cancer and cancer in situ. We found that the invasive component expressed high levels of extracellular matrix-associated genes. The six cancer-in-situ regions were surprisingly heterogeneous, suggesting that the biopsy contained multiple individual cancer clones evolving in parallel. This information would not have been available with regular cancer diagnostic tools or sequencing technology.

6.5.2 Discussion (Paper IV)

RNA sequencing is very useful for classifying, cataloging and characterizing the cell types that exist in the body. The method presented in Paper IV introduces a new dimension to gene expression analysis because of its spatial aspect. Using this technique, distinct cell types and states that are detected can be placed in their right spatial context, adding very useful information to the analysis.

The resolution of spatial transcriptomics depends on the size and distribution of barcode-coated features arrayed on the chip. Currently, each such feature is 100 μm in diameter, and the center-to-center distance of neighboring features is 200 μm. This means that the current version of spatial transcriptomics does not have single-cell resolution and that there are areas between the features where RNA is not captured.

The detection limit of transcripts is on the lower end of that reported for single-cell RNA sequencing, detecting 6.9% of the number of transcripts smFISH detected from a control sample. Together with the fact that average sequencing read length was around 300-600 nucleotides, this means spatial transcriptomics is currently most suitable to detect spatial differences in transcripts expressed at medium to high levels and that it is suitable for analyses that do not require full-length transcripts.

Taken together, the spatial dimension introduced by spatial transcriptomics makes it a powerful tool, useful both basic research and clinical diagnostics. Current limitations to the method's spatial resolution and sequencing depth make the method suitable for studying clear transcriptional differences between broad spatial regions.

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